

2014

A novel cytostatic form of autophagy in sensitization of non-small cell lung cancer cells to radiation by vitamin D and vitamin D analogue, EB 1089.

khushboo sharma
sharmak3@vcu.edu

Follow this and additional works at: <http://scholarscompass.vcu.edu/etd>

© The Author

Downloaded from

<http://scholarscompass.vcu.edu/etd/3569>

This Dissertation is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.

A NOVEL CYTOSTATIC FORM OF AUTOPHAGY IN SENSITIZATION OF NON-
SMALL CELL LUNG CANCER CELLS TO RADIATION BY VITAMIN D AND THE
VITAMIN D ANALOGUE, EB 1089.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at
Virginia Commonwealth University.

By

KHUSHBOO SHARMA

Master of Science, Virginia Commonwealth University, 2010

Director: DAVID A. GEWIRTZ, PH.D

PROFESSOR, PHARMACOLOGY AND TOXICOLOGY

Virginia Commonwealth University

Richmond, Virginia

September, 2014

Acknowledgements

I would like to express my most sincere gratitude to everyone who assisted me throughout my dissertation project. My time at the Virginia Commonwealth University has been so valuable to me due to the support and guidance of the great people I've been surrounded by. My deepest gratitude to Dr. David Gewirtz for his guidance, patience, and thoughtful discussion that has helped me grow and expand my skills as a scientist. I would also like to thank to my committee members, Dr. Jill Bettinger, Dr. Hisashi Harada, Dr. Steven Grant, and Dr. Joseph Ritter for their time and advice.

I have been extremely lucky to work with an excellent group of people in the Gewirtz lab. I am grateful to all the past and present members of the Gewirtz lab for their kindness and laughs throughout the years. I am especially thankful to Dr. Rachel Goehe for mentoring me. Her guidance and support were instrumental in my growth as a scientist.

Lastly, I would like to thank my friends and family back home for their long-distance encouragement, especially my mother, Indu Sharma and my father, Satish Sharma, for believing in me. I wish to extend a special thanks to my friends, Soumya warrier, Shilpa Singh, Shweta Chakradeo, and Ngoc Le, for their immense support and friendship throughout, they are amazing and I wouldn't have survived graduate school without them.

Table of contents

	Page
Acknowledgements.....	ii
List of Figures.....	vi
List of tables.....	xii
List of abbreviations.....	xiii
List of contributions.....	xiv
Abstract.....	xv
 Chapter	
1 Introduction.....	1
1.1.Cancer.....	1
1.2.Lung Cancer.....	3
1.3.Treatment options.....	5
1.3.1.Surgery.....	5
1.3.2.Chemotherapy.....	5
1.3.3.Targeted treatments.....	6
1.3.4.Radiation.....	6
1.4.Responses to therapies.....	10
1.4.1.Apoptosis (and Necrosis).....	10
1.4.2.Senescence.....	12
1.4.3.Radiation-induced cell cycle arrest.....	15
1.4.3.Autophagy.....	17
1.5.Multiple forms/functions of Autophagy.....	22
1.5.1.Cytoprotective Autophagy.....	24
1.5.2.Non-protective Autophagy.....	25
1.5.3.Cytotoxic Autophagy.....	26

1.6.	Signaling pathways associated with autophagy.....	28
1.6.1.	AMPK/ULK-1 pathway.....	28
1.6.2.	p53 and its role in autophagy.....	32
1.7.	Vitamin D.....	36
1.7.1.	Vitamin D and Autophagy.....	38
1.7.2.	Vitamin D and Cancer.....	39
1.8.	Previous studies.....	41
1.9.	Cytostatic Autophagy.....	44
2	Methods and materials.....	48
2.1.	Cell lines.....	48
2.2.	Cell culture and treatment.....	49
2.3.	Cell viability and clonogenic survival.....	50
2.4.	FACS analysis for Annexin V and Propidium Iodide positive cells to assess apoptosis.....	50
2.5.	Detection and quantification of senescent cells.....	51
2.6.	Detection and quantification of acidic vacuoles with acridine orange staining.....	52
2.7.	GFP-LC3 redistribution.....	53
2.8.	Western Blotting.....	53
2.9.	Assessment of kinase phosphorylation and γ H2Ax Formation.....	54
2.10.	Statistical Analysis.....	55
3	Sensitization to radiation by vitamin D.....	56

3.1. Influence of 1,25-D ₃ and vitamin D analogue EB 1089 on sensitivity to radiation in non-small cell lung cancer cells.....	56
3.2. Vitamin D sensitizes lung tumor cells to radiation.....	63
3.3. Sensitization limited to cells with wt/functional p53 and VDR.....	66
3.4. Summary.....	68
4 Identifying the basis of radio-sensitization.....	69
4.1. 1,25-D ₃ and vitamin D analogue EB 1089 fail to increase the extent of DNA damage or interfere with DNA repair in non-small cell lung cancer cells.....	69
4.2. Minimal induction of apoptosis or necrosis by radiation and radiation + 1,25-D ₃ / EB 1089 in NSCLC cells.....	70
4.3. 1,25-D ₃ /EB 1089 fail to increase the extent of senescence induction by radiation in NSCLC cells.....	77
4.4. Evidence that autophagy is the mode of radio-sensitization by 1,25-D ₃ /EB 1089.....	83
4.5. Autophagy induced by radiation treatment is cytoprotective in nature.....	93
4.6. Increased sensitivity to the combination treatment of IR + EB 1089 is associated with a novel form of autophagy.....	104
4.7. Effects of radiation and EB 1089 + radiation are associated with cell cycle arrest.....	114
4.8. Effects of 1,25-D ₃ and EB 1089 on radiation sensitivity in normal cells.....	116
4.9. Summary.....	118
5 Exploring the signaling pathways involved in vitamin D mediated radio-sensitization.....	120
5.1. p53's involvement in radio-sensitization by vitamin D.....	120

	5.2. The AMPK signaling pathway.....	123
	5.3. Summary.....	144
6	Discussion.....	145
	6.1. Sensitization to radiation.....	147
	6.2. Radiation treatment alone induces cytoprotective function of autophagy.....	149
	6.3. Radio-sensitization by vitamin D caused by a change in the function of autophagy.....	150
	6.4. Efforts to identify signaling pathways associated with autophagy.....	152
	6.5. Autophagy and senescence relationship?.....	156
	6.6. Clinical trials.....	157
	6.7. Future studies.....	158
	6.8. Summary.....	162

List of figures

Figure 1.1: DNA damage induced by radiation.....	9
Figure 1.2: Detailed Extrinsic and Intrinsic pathways involved in Apoptosis.....	12
Figure 1.3: Hallmarks of Senescence.....	14
Figure 1.4: Cell cycle of eukaryotic cells.....	16
Figure 1.5: Different stages of the autophagic process.....	18
Figure 1.6: Different types of autophagy.....	20
Figure 1.7: Different functions of autophagy induced in response to different Stresses and cell type.....	27
Figure 1.8: Comprehension of different functions of autophagy upon autophagy Inhibition.....	27
Figure 1.9: Role of AMP-activated protein kinase in the control of autophagy.....	31
Figure 1.10: Proposed model for autophagy signaling pathway in response to radiation in NSCLC.....	35
Figure 1.11: Vitamin D metabolism.....	37
Figure 1.12: A novel function, cytostatic, identified in NSCLC cells.....	45
Figure 3.1: Vitamin D receptor status in NSCLC cell lines.....	59
Figure 3.2: Radiation dose selection.....	59
Figure 3.3: Modest effects of 1,25-D ₃ or EB 1089 on cell survival.....	60
Figure 3.4: Modest effects of 1,25-D ₃ or EB 1089 on cell viability.....	61
Figure 3.5: Dose response curve for the combination treatment of IR and EB 1089.....	62
Figure 3.6: Radiosensitization of NSCLC cells by 1,25-D ₃ /EB 1089.....	64
Figure 3.7: 1,25-D ₃ /EB 1089 increases radiosensitivity and inhibits proliferative recovery of NSCLC cells.....	65

Figure 3.8: Lack of sensitization in p53 mutant and VDR lacking NSCLC cells.....	67
Figure 4.1: Induction and repair of DNA damage by IR and 1,25-D ₃ /EB 1089 + IR.....	72
Figure 4.2: Assessment of apoptosis (and necrosis) induction in A549 cells by flow cytometry.....	73
Figure 4.3: Minimal increase in apoptosis in response to radiation sensitization in A549 cells.....	74
Figure 4.4: Assessment of apoptosis (and necrosis) induction in H460 cells by flow cytometry.....	75
Figure 4.5: Minimal increase in apoptosis in response to radiation sensitization in H460 cells.....	76
Figure 4.6: Assessment of senescence induction by radiation and radiation + 1,25-D ₃ in A549 cells.....	79
Figure 4.7: No significant increase in senescence by IR + 1,25-D ₃ compared to IR in A549 cells.....	80
Figure 4.8: Assessment of senescence induction by radiation and radiation + EB 1089 in H460 cells.....	81
Figure 4.9: No significant increase in senescence by IR + EB 1089 compared to IR in H460 cells.....	82
Figure 4.10: Analysis of autophagy induction by acridine orange staining in A549 NSCLC cells.....	87
Figure 4.11: Assessment of autophagy by flow cytometry in A549 NSCLC cells.....	88
Figure 4.12: Assessment of autophagic flux in A549 NSCLC cells.....	89
Figure 4.13: Analysis of autophagy induction by acridine orange staining in H460 NSCLC cells.....	90

Figure 4.14: Assessment of autophagy by flow cytometry in H460 NSCLC cells.....	91
Figure 4.15: Assessment of autophagic flux in H460 NSCLC cells.....	92
Figure 4.16: Dose response with autophagy inhibitors Chloroquine and Bafilomycin A1	95
Figure 4.17: Minimal effect of autophagic inhibitors on cell viability.....	95
Figure 4.18: Inhibition of radiation induced autophagy in A549 cells.....	96
Figure 4.19: Cytoprotective autophagy induced by radiation in A549 cells.....	97
Figure 4.20: Inhibition of radiation induced autophagy in H460 cells.....	98
Figure 4.21: Cytoprotective autophagy induced by radiation in H460 cells.....	99
Figure 4.22: Assessment of apoptosis upon inhibition of autophagy in A549 cells by flow cytometry.....	100
Figure 4.23: Induction of apoptosis upon inhibition of autophagy in A549 cells.....	101
Figure 4.24: Assessment of apoptosis upon inhibition of autophagy in H460 cells by flow cytometry.....	102
Figure 4.25: Induction of apoptosis upon inhibition of autophagy in H460 cells.....	103
Figure 4.26: Evidence for inhibition of autophagy by chloroquine and bafilomycin A1 in H460 cells.....	107
Figure 4.27: Evidence that radiosensitization by EB 1089 occurs through a cytostatic function of autophagy.....	108
Figure 4.28: Knockdown of autophagy related genes, <i>ATG5</i> and <i>BECN1</i> , in H460 cells.....	109

Figure 4.29: Inhibition of autophagy by genetic silencing of <i>ATG5</i> and <i>BECN1</i> in H460 cells.....	109
Figure 4.30: Genetic knockdown of <i>ATG5</i> and <i>BECN1</i> reverses EB 1089 induced radiosensitization.....	110
Figure 4.31: EB 1089 causes a change in function of autophagy as indicated upon autophagy inhibition by knockdown of <i>ATG5</i> gene.....	111
Figure 4.32: EB 1089 causes a change in function of autophagy as indicated upon autophagy inhibition by knockdown of <i>BECN1</i> gene.....	112
Figure 4.33: Influence of genetic silencing of autophagy on apoptosis induction by IR and IR+EB 1089 in H460 cells.....	113
Figure 4.34: Cell cycle analysis.....	115
Figure 4.35: No difference in sensitization by IR compared to IR+1,25-D ₃ in cardiomyocytes.....	117
Figure 4.36: No difference in sensitization by IR compared to IR+1,25-D ₃ in human bronchial epithelial cells	117
Figure 5.1: Induction of p53 in H1299 cells indicated by western blotting.....	124
Figure 5.2: Lack of radiosensitization by EB 1089 in p53 null (-Dox) H1299 cells.....	125
Figure 5.3: Radiosensitization by EB 1089 in p53 induced (+Dox) H1299 cells.....	126
Figure 5.4: Autophagy induction in p53 null (-Dox) H1299 cells.....	127
Figure 5.5: Autophagy induction in p53 induced (+Dox) H1299 cells.....	128
Figure 5.6: Inhibition of autophagy in p53 null (-Dox) H1299 cells by chloroquine.....	129
Figure 5.7: Inhibition of autophagy in p53 inducible (+Dox) cells by chloroquine.....	130
Figure 5.8: Inhibition of autophagic flux in H1299 cells by western blotting.....	131

Figure 5.9: Blocking autophagy reverses the sensitization in p53 inducible (+Dox) H1299 cells.....	132
Figure 5.10: Genetic knockdown of autophagy genes in H1299 cells.....	133
Figure 5.11: Autophagy inhibition in p53 null (-Dox) H1299 cells by knockdown of autophagy related genes.....	134
Figure 5.12: Autophagy inhibition in p53 inducible (+Dox) H1299 cells by knockdown of autophagy related genes.....	135
Figure 5.13: Blocking autophagy by knocking down <i>BECN1</i> reverses the sensitization in p53 inducible (+Dox) H1299 cells.....	136
Figure 5.14: Blocking autophagy by knocking down ATG5 reverses the Sensitization in p53 inducible (+Dox) H1299 cells.....	137
Figure 5.15: Induction in phosphorylation of AMPK in response to IR and IR+EB 1089 treatments.....	138
Figure 5.16: Assessment of changes in AMPK phosphorylation by flow cytometry.....	139
Figure 5.17: Inhibition of phosphorylation of AMPK and ACC by compound C.....	140
Figure 5.18: Minimal effect of compound C on cell viability.....	141
Figure 5.19: Assessment of GFP-LC3 redistribution after inhibition of AMPK using compound C.....	142
Figure 5.20: Effects of pharmacological inhibition of AMPK on cell viability.....	143

List of Tables

Table 1.1: Summary of characteristics of different functions/forms of autophagy.....	46
--	----

List of Abbreviations

1,25-D3	1,25 dihydroxyvitamin D3
DAPI	4',6-diamidino-2-phenylindole
AMPK	5' adenosine monophosphate-activated protein kinase
AO	Acridine orange
ATCC	American type culture collection
AVOs	Acidic vacuolar organelles
CQ	Chloroquine
EB 1089	Secocalcitol or 22E,24E-Diene-24,26a,27a-trihomo-1a,25(OH) ² -vitaminD3
FACS	Fluorescence-activated cell sorting
GFP	Green fluorescent protein
Gy	Gray
IR	Ionizing radiation
LC3	Microtubule associated protein light chain 3
PI	Propidium Iodide
RXR	Retinoic X receptor
Baf	Bafilomycin A1
ULK1	unc-51- like kinase 1
VDR	Vitamin D receptor
VDRE	Vitamin D response element

List of Contributions

All the experiments included in this dissertation were performed by Khushboo Sharma except studies shown in figure 3.3 (upper panel), figure 3.8 (lower panel) and figure 4.36 (upper panel), which were contributions of Dr. Rachel Goehe and Dr. Xu Di.

Abstract

A NOVEL CYTOSTATIC FORM OF AUTOPHAGY IN SENSITIZATION OF NON-SMALL CELL LUNG CANCER CELLS TO RADIATION BY VITAMIN d AND THE VITAMIN D ANALOGUE, EB 1089.

By Khushboo Sharma, PhD

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2010

Major Director: David A Gewirtz, Ph.D.

Professor, Pharmacology and Toxicology

The standard of care for unresectable lung cancer is chemoradiation. However, therapeutic options are limited and patients are rarely cured. While Radiation therapy is effective at killing tumor cells or inhibiting their growth initially, development of resistance to treatments and recurrence of tumors are major issues. One of the major goals of Dr. Gewirtz's laboratory has been to develop strategies to overcome the resistance and attenuate disease recurrence. One of these attempts involve employing

vitamin D and its analogs in combination with radiation therapy. Our proposed studies were based on a previous finding where vitamin D and vitamin D analogs such as EB 1089, were shown to enhance the response to radiation in breast cancer through the promotion of autophagy.

We extended these studies to non-small cell lung cancer (NSCLC) and were able to validate that 1,25-D₃ (the hormonally active form of vitamin D) and EB 1089 does in fact sensitize A549 and H460 cells and prolonged the growth arrest induced by radiation alone and suppressed proliferative recovery, which translated to a significant reduction in clonogenic survival. In H838 or H358 NSCLC cells, which lack the vitamin D receptor or functional p53, respectively, 1,25-D₃ failed to modify the extent of radiation-induced growth arrest or suppress proliferative recovery post irradiation. Sensitization to radiation in H1299 NSCLC cells was evident only when p53 was induced in otherwise p53 null H1299 NSCLC cells. Sensitization by 1,25-D₃/ EB 1089 was not associated with increased DNA damage, decreased DNA repair or an increase in apoptosis, necrosis or senescence. Instead sensitization appeared to be a consequence of the conversion of the cytoprotective autophagy induced by radiation alone to a novel cytostatic form of autophagy by the combination of 1,25-D₃ or EB 1089 with radiation. While both pharmacological and genetic suppression of autophagy or inhibition of AMPK phosphorylation sensitized the NSCLC cells to radiation alone, inhibition of the cytostatic autophagy induced by the combination treatment reversed sensitization. Evidence for selectivity was provided by lack of radiosensitization in normal human bronchial cells and cardiomyocytes. Taken together, these studies have identified a unique cytostatic function of autophagy that appears to be mediated by the vitamin D receptor, p53 and possibly AMPK in the promotion of an enhanced response to radiation by 1,25-D₃ and EB 1089 in NSCLC.

Chapter 1

Introduction

1.1 Cancer:

The term, “cancer” is derived from Karkinos, which means crab in the Greek language. Hippocrates (460-370 BC) coined the term “cancer”, which stands for malignant tumors, where cells grow abnormally and are not under the control of normal internal and external regulatory mechanisms, (Brucher BL, *et al.* 2014). The oldest known case of metastatic cancer was discovered in a 2,700 year- old male skeleton in Siberia, Russia. Not all tumors are cancerous; some can be benign, which are not life threatening since they do not invade or metastasize to other tissue sites; however these tumors can grow quite large and press on other healthy cells, organs and tissues (American Cancer Society, T. 2014d).

Cancer is one of the most common causes of death in the United States as well as other parts of the world. One in 4 deaths in the United States is due to cancer. As per 2012 statistics, estimated new cases and estimated deaths due to cancer

are 1,638,910 and 577,190 respectively (Siegel R, *et al.* 2012). Efforts at eradicating cancer have been ongoing, largely since President Nixon took the very first major effort in 1971 by signing the National Cancer Act, with the primary objective of strengthening the National Cancer Institute. Since that time billions of dollars have been spent on cancer research, data collection and analysis in the USA alone.

The most common alterations that occur in cancer are: self-sufficiency in growth signals, insensitivity to growth inhibitory signals, sustained angiogenesis, tissue invasion and metastasis, evasion of programmed cell death, and limitless replicative potential (Hanahan D and Weinberg, 2000). Drug resistance and the ability of cancer cells to grow even when one pathway is blocked have made the initial treatments such as surgery, chemotherapy, and using radiation alone less effective or completely ineffective (Dent P, *et al.* 2003).

Cancer epidemiology has become an important field of study where researchers are working towards gathering information regarding the factors that might increase the risk of cancer such as immoderate tobacco use, excessive smoking, UV radiation and factors which can help prevent/stop cancer such as healthy lifestyle, exercise etc. These findings are important from a public health point of view (American Cancer Society, T. 2014d) and provide insight into the interaction of genes at the molecular level with external cancer causing factors (American Cancer Society, T. 2014d).

It was in the 20th century after DNA was discovered that scientist realized that it was damage to the DNA structure by various chemicals or radiation, or introduction of a new DNA sequence by viruses, which often were the causes of tumor formation. Under normal circumstances, any significant degree of damage to DNA would likely cause normal cell to die or undergo repair; however some mutations (in oncogenes and/or tumor suppressor genes, see below) are beneficial to the cells in that they do not die but develop into tumor cells that continue to grow indefinitely (American Cancer Society, T. 2014d).

1.2 Lung Cancer:

Lung cancer is the most common cancer in the world and second most commonly diagnosed cancer in the United States (Staintigny P, *et al.* 2012). As per statistics, estimated new cases and deaths due to lung cancer in the United States in 2014 are expected to be 224,210 and 159,260 (National Cancer Institute, T. 2014a; Siegel R, *et al.* 2012).

Lung cancer can broadly be divided into Small cell lung cancer (SCLC) and Non-small cell lung cancer (NSCLC). Small cell lung cancer is identified morphologically by its small round nodule shape appearance under the microscope. This type of cancer is more common in the smoking population compared to non-smokers and spreads much faster compared to non-small cell lung cancer. The best treatment course for small cell lung cancer is usually chemotherapy; the most commonly used drugs are cisplatin in combination with paclitaxel or gemcitabine and/or radiation if the cancer has spread throughout the

body (Johnson DH, *et al.* 2008). However since 85% of lung cancers are NSCLC, which have poor prognosis and are aggressive in nature, we have focused this study mainly on sensitization of NSCLC cell lines.

Non-small cell lung cancer can be divided into 3 main types (American Cancer Society, T. 2014a)-

A. Squamous cell carcinoma- this type of cancer is also known as epidermoid carcinoma and it is the second most common subtype of NSCLC, comprising up to 25% of NSCLC cases in the United States. This type of NSCLC usually originates in the center of the lung in the bronchi and often stays within the lung, spreads to lymph nodes and forms a cavity within the lung. Smoking is one of the major causes of this cancer.

B. Adenocarcinoma – this is the most common type of NSCLC contributing to 40% of NSCLC cases in the United States. This particular type originates in the outer layers of the lung; it develops from the cells lining the air passages and can spread to lymph nodes and even beyond. A subtype of adenocarcinoma, bronchiolo alveolar carcinoma (BAC), develops at multiple sites in the lungs and spreads to preexisting alveolar walls.

C. Large cell carcinoma- this type of cancer is the least common and accounts for only 10-15% of the total NSCLC cases in United States. This type is also commonly known as undifferentiated carcinomas.

Others:

Bronchial carcinoids: this type accounts for up to 5% of lung cancers. These tumors can metastasize and a small proportion of these tumors can secrete

hormone like substances. These tumors are usually small, spread and grow slowly. This type of cancer can be removed surgically when detected at relatively early stages.

1.3 Treatment options:

Different treatment options available for NSCLC are surgery, chemotherapy, radiation, and targeted therapy (American Cancer Society, T. 2014b). These treatments are mainly based on tumor histology, extent of advancement (stage), and patient specific personal factors (lung functionality, morbidity etc.) (Baltayiannis, N. *et al.* 2013).

1.3.1 Surgery:

Surgical therapy remains the keystone in NSCLC treatment in order to offer long-term survival after a rigorous selection in terms of patients with early stage and late more advanced stage cancer. Surgical techniques have progressed tremendously in recent years (Baltayiannis, N. *et al.* 2013). Most stage I and II NSCLC are treated with surgery to remove the tumor, usually lobectomy, where a lobe or a section of lung containing tumor is removed selectively.

1.3.2 Chemotherapy:

After surgical removal of the cancer, patients are usually treated with chemotherapy to avoid recurrence; this is known as 'adjuvant chemotherapy' and is usually done for patients with early cancers. The chemotherapy treatment generally involves cisplatin or carboplatin in combination with gemcitabine or docetaxel (American Cancer Society, T. 2014b). When these drugs are not

effective in the first cycle of treatment, a second round is prescribed, usually with a single drug or targeted therapy drug, which is known as second-line chemotherapy

1.3.3 Targeted treatments: These treatments involve using drugs which are specific to cancer cells and do not target normal/healthy cells in the surrounding tissue (American Cancer Society, T. 2014b). These drugs specifically attach to or block targets that are present on the surface of tumor cells. Some of the relatively well-established drugs used for targeted therapy in NSCLC are erlotinib, gefitinib, which target the tyrosine kinase domain of epidermal growth factor receptor (EGFR), and bevacizumab,, which targets vascular endothelial growth factor (VEGF) (American Cancer Society, T. 2014b).

1.3.4 Radiation:

For patients with advanced stages of lung cancer where surgery cannot be used to remove the tumor, high doses of radiation are used in combination with chemotherapy. This is usually the case for stage III cancers. At times, receiving chemotherapy and/or radiation before surgery can help shrink the tumor and make it easier to remove. This is done in order to make the treatment more effective (National Cancer Institute, T. 2010a).

Radiation treatment can be given either externally or internally (American Cancer Society; 2014c). External beam radiation therapy uses an external source of radiation, which directs radiation to a particular part of the body where the tumor is growing whereas the internal method known as brachytherapy involves using a radioactive material, which is placed inside the body. The radiation rays from this

material can only travel short distances, thereby not harming the surrounding normal tissue (National Cancer Institute, 2007a).

The amount of radiation used in radiation therapy is measured in gray (Gy) units and the dose is decided based on the histology, type and stage of lung cancer (National Cancer Society 2014a). Typically, 60-70Gy is used for treating non-small-cell lung cancers and patients receive fractionated doses of 1.8-2Gy five times a week over a span of 5-6 weeks (American cancer society, 2014c). Fractionated dosing allows the adjoining normal cells to recover from damage in case radiation is not very focused (National Cancer Institute T. 2010a). Since normal cells generally proliferate more slowly than cancer cells, this provides more time to repair the damage (Begg AC, *et al.* 2011). Patients with inoperable locally advanced tumors are the primary candidates for radiation therapy (National Cancer Institute, T. 2010a).

Radiation destroys tumor cells by causing damage to their DNA either directly or indirectly by creating charged particles (reactive oxygen species) that leads to oxidative stress (Chen DJ and Nirodi CS, 2007). Radiation can cause a number of different types of DNA damage such as single-strand breaks, double-strand breaks, DNA adducts, and DNA-protein cross-links (Hall and Giaccia, 2004). The most lethal lesions produced are double-strand breaks since they cannot be repaired as easily by the DNA repair mechanisms (Spitz DR, *et al.* 2004). Indirect DNA damage occurs by the charged particles such as hydroxyl (OH) and O₂ radicals formed by ionization of water (Hall and Giaccia, 2006).

Cells can undergo a number of responses to radiation induced DNA damage such as cell cycle arrest, senescence, apoptosis, and autophagy (Sun JG, et al. 2011; Luo H, et al. 2013; Kim KW, et al. 2011; Sancar A, et al. 2004). Depending on the type and extent of damage induced by radiation, a number of proteins are recruited and activated at the site of damage such as the MRN complex (consists of DNA repair proteins; Mre11, Rad50 and Nbs1) that detects double strand breaks and the RPA protein (Replication protein A; a heterotrimeric, single-stranded DNA-binding protein) that detects single strand breaks (Uziel T, et al. 2003; Flack J, et al. 2005). MRN contributes to recruitment and activation of ATM (Ataxia telangiectasia mutated), which is a serine/threonine kinase (Uziel T, et al. 2003; Flack J, et al. 2005), while RPA recruits ATR (Ataxia telangiectasia related) kinase. Once activated, these kinases can further activate effector kinases chk1 and chk2, which spread the signal further. This leads to a temporary growth arrest where cells make an attempt to repair the damage; if the damage is repaired, then they cells can reenter the cell cycle and resume proliferation (Sancar A, et al. 2004). If the damage cannot be repaired due to compromised repair mechanisms or excessive damage then it is thought that cells undergo cell death such as apoptosis or necrosis (Elmore S. 2007).

As with many other therapeutic approaches, radiation also has some limitations. Even though initial treatment with radiation is quite effective in destroying tumor cells or suppressing their growth, there are factors that lead to development of resistance to radiation treatment such as refractoriness to apoptosis in the absence of p53 (Zheng R *et al.* 2014). Recurrence of cancer due to resistance to

several treatments such as radiation, chemotherapy, and surgery is one of the major issues that need to be addressed in order to attain an effective cancer treatment. One of the major issues with non-small cell lung cancer is that it is often diagnosed at quite a late stage when tumors have grown large and lie in close proximity to normal tissues. Due their close association with the other normal tissue, high doses of radiation cannot be used to treat NSCLC. Therefore, there is a need to develop strategies to sensitize these tumors to moderate doses of radiation. In two of the largest retrospective radiation therapy studies, patients with inoperable disease treated with definitive radiation therapy (external beam radiation therapy) achieved over all 5-year survival rates of only 10% and 27% with median survival being 19.5 and 27.9 months respectively (Dosoretz DE, *et al.* 1992; Gauden S, *et al.* 1995).

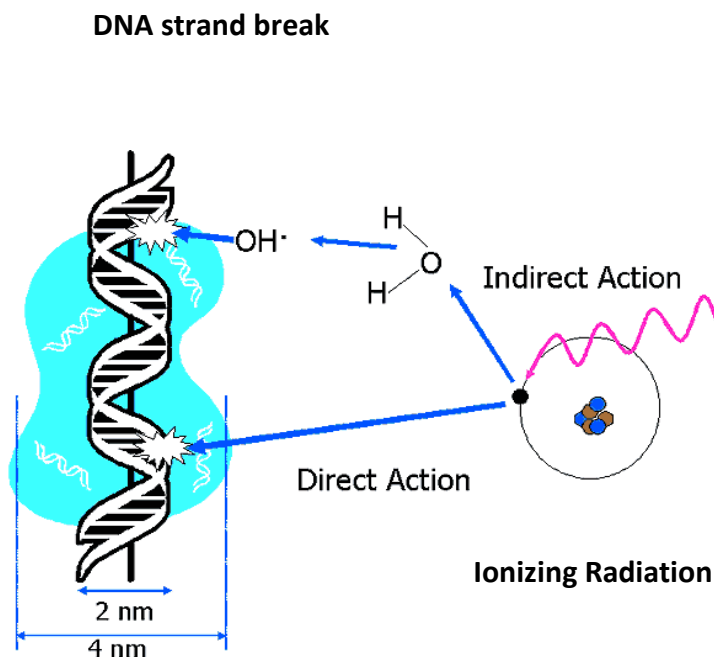


Figure 1.1 DNA damage induced by radiation.

Radiation can cause damage either directly by causing double-strand breaks or create charged particles and cause damage indirectly. Dosoretz DE, *et al.* 1992

1.4 Responses to therapies

1.4.1 Apoptosis (and Necrosis)

Apoptosis is also known as programmed cell death I. It is an intracellular death program that becomes activated when cells are to be eliminated by normal biological processes (Elmore S. 2007). In a typical apoptosis process, a cell shrinks, condenses, the nuclear envelope disassembles, the cytoskeleton collapses, and DNA breaks up into fragments (Boe R, et al. 1991). In some forms of acute injury leading to sudden death, cells may swell up and burst open spilling their cellular contents onto the neighboring cells. This process is called necrosis and leads to release of chemotactic signals, which eventually recruit inflammatory cells (Elmore S. 2007, Kurosaka K *et al.* 2003). In apoptosis, however, there isn't any inflammatory reaction.

Intracellular machinery responsible for apoptosis appears to be similar in all biological systems. This process uses a class of proteases, called caspases, which have a cysteine group at their active site and cleave their target group at specific sites, which have aspartic acid (Mcllwain DR, et al. 2013). These caspases usually exist in their inactive precursor form known as pro-caspases; once activated by aspartic acid cleavage, they further cleave and activate other caspases resulting in an amplified cascade process (Mcllwain DR, et al. 2013). Pro-caspase activation is triggered by adaptor proteins, which bring multiple copies of initiator pro-caspases close together to form an aggregate, which undergoes a conformational change to activate procaspase. Once the caspase at

the top of the cascade is activated, it cleaves downstream procaspases and amplifies the death signal and spread it throughout the cell (McIlwain DR, et al. 2013).

The mechanisms of apoptosis are highly sophisticated and complex, as shown in the figure above (Chipuk JE, *et al.* 2006). The two main pathways are: the extrinsic pathway and the intrinsic pathway (Chipuk JE, et al. 2006). Evidence suggests that these pathways might be linked and influencing each other (Igney FH, *et al.* 2002). The extrinsic pathway involves activation of death receptors after binding to ligands such as FasL, TNF-alpha, Apo3L, and Apo2L (Fesik SW 2005). These death receptors further activate caspase8, caspase7, capase3 and lead to cell death. The Intrinsic pathway involves members of Bcl-2 family proteins, which cause mitochondrial outer membrane permeabilization to release cytochrome C leading to cell death by activating caspase 3 (Chipuk JE *et al.* 2006; Elmore S *et al.* 2007)

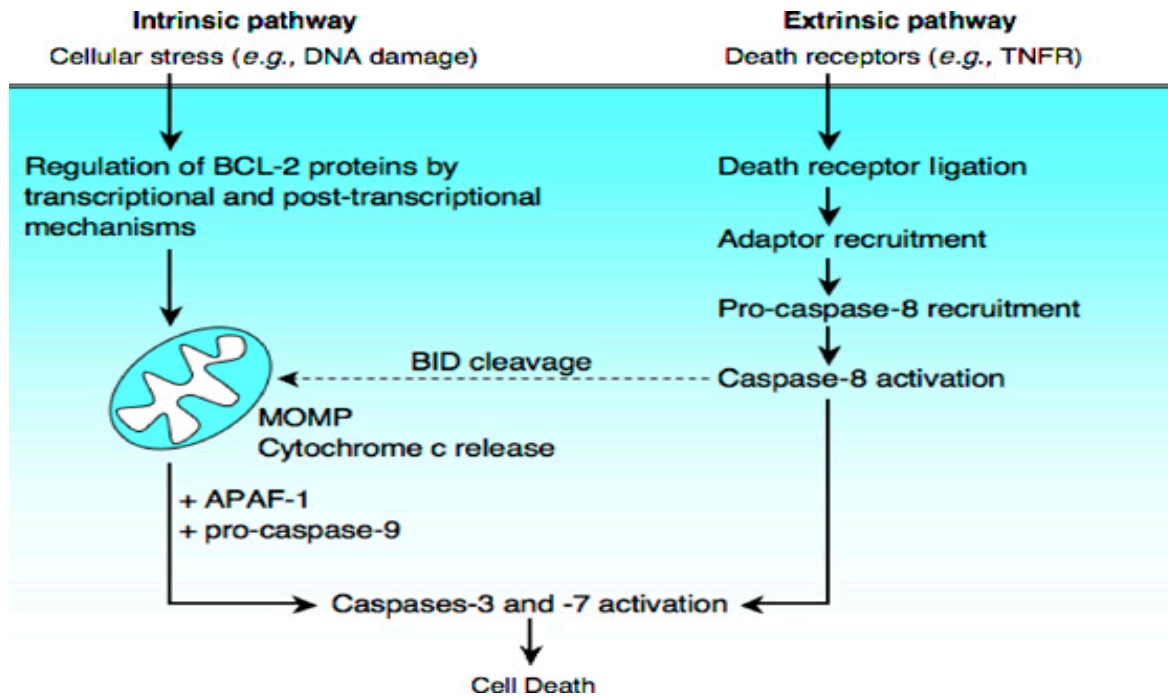


Figure1.2 Detailed Extrinsic and Intrinsic pathways involved in Apoptosis. The extrinsic pathway involves activation of death receptors after binding to ligands such as TNF-alpha and The Intrinsic pathway involves members of Bcl-2 family proteins, which cause mitochondrial outer membrane permeabilization to release cytochrome C leading to cell death by activating caspase 3 (Chipuk JE et al. 2006).

1.4.2. Senescence

Senescence is a biological phenomenon in which cells lose their ability to divide but are still metabolically active. It was first discovered and described more than 40 years ago by Hayflick and Moorhead who linked cellular senescence to both aging and tumor suppression (Hayflick L and Moorhead PS, 1961; Rodier F, *et al.* 2011). Recent literature now suggests that senescence can even contribute to normal tissue repair and tumor advancement (Rodier F, *et al.* 2011). The three major identified forms of senescence are: Oncogene-induced senescence, replicative senescence and premature (accelerated or stress induced)

senescence (Gewirtz *et al.* 2008). Oncogene-induced senescence occurs in cells in the presence of the expression of oncogenes such as RAS, evidently in order to delay transformation (Lee AC, *et al.* 1999). Expression of V12RAS has been linked to increased ROS levels (Lee AC, *et al.* 1999), which can induce DNA damage and result in oxidative modifications of DNA, further leading to up-regulation of p53 and p21 which induce growth arrest in order to repair the damage (Lee AC, *et al.* 1999). Replicative senescence reflects the ability of cells to undergo senescence caused by telomere shortening, which occurs after multiple numbers of cell divisions in non-transformed cells (Shay and Robinson, 2004). Premature, accelerated or stress induced senescence occurs when cells undergo stress due to exogenous cytotoxic agents, which usually cause DNA damage (Gewirtz, *et al.* 2008).

Some of the salient features of senescence include: A) Permanent growth arrest, usually irreversible by any known physiological stimuli. However as p16INK4a is required to maintain this irreversibility, cells that lack p16 can resume growth upon genetic interventions (Beausejour *et al.* 2003). B) Induced expression of senescence associated beta-galactosidase due to increased lysosomal mass (Dimri GP, *et al.* 1995; Lee BY, *et al.* 2006). C) Changes in cell morphology. Cells increase in size, become larger and more flattened (Hayflick L. 1965). D) Increased levels of p16INK4a, which activates the tumor suppressor, Rb, and causes an increase in formation of senescence associated heterochromatin foci (SAHF) (Narita M, *et al.* 2003). SAHF also serves as a marker for identification of senescence.

Senescence activation is also linked to secretion of senescence associated secretory proteins (SASP). The cells release a complex mixture of extra cellular matrix, soluble factors and proinflammatory cytokines such as IL6 and IL8 and are usually released as a part of an innate immune response responsible for tumor clearance (Xue W, *et al.* 2007).

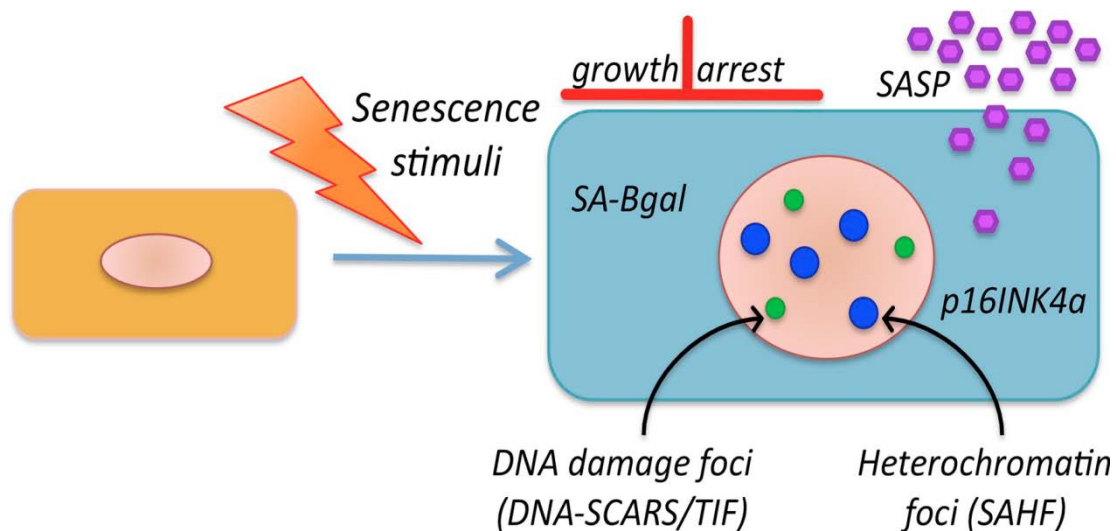


Figure 1.3 Hallmarks of senescence: These hallmarks include expression of SA-Bgal and p16INK4a; robust secretion of numerous growth factors, cytokines, proteases, and other proteins (SASP); and nuclear foci containing DDR proteins (DNA-SCARS/TIF) or heterochromatin (SAHF). (Rodier F and Campisi JJ. *Cell Biol*; 2011)

1.4.3 Radiation-induced cell cycle arrest

A number of proteins are generally activated when cells sense DNA damage. p53 is central to the cells' response to DNA damage (Cheng G *et al.* 2013). p53 is known as 'guardian of the genome' and can inhibit tumorigenesis by inducing cell cycle arrest to allow for DNA damage to repair (Cheng G, *et al.* 2013).

Compared to normal cells, cancerous cells can generally divide much more rapidly (Collins K, *et al.* 1997). The process of cell division usually involves well-coordinated and orchestrated events that are together known as the cell division cycle, as shown in the figure 1.4. Each cell cycle consists of essentially 4 phases; S-phase (DNA Synthesis), M-phase (Mitosis), G1-phase (Gap-1), G-2 phase (Gap-2). There is an additional phase that cells can enter when they stop cycling after division known as the G0 phase, a state of quiescence (Collins K, *et al.* 1997). The process of DNA replication and cell division is tightly regulated to ensure proper cell growth and prevent replication of damaged DNA (Gogineni VR, *et al.* 2011). A family of CDK-cyclin complexes plays a critical role in keeping this process in check. A series of phosphorylation and dephosphorylation events regulate the activity of these kinases, ensuring well-defined transitions between cell cycle stages (Morgan DO. 1995, Collins K, *et al.* 1997).

Various cellular stresses can lead to growth arrest. Cell cycle progression can be halted in response to growth factor withdrawal, contact inhibition, and radiation-induced DNA damage (Gogineni VR, *et al.* 2011). A set of checkpoint controls maintains the orderly nature of cell cycle process. These checkpoints, at the

transition between G1/S and G2/M phases, sense any kind of faults in critical events such as chromosome segregation and DNA replication (Elledge SJ. 1996; Wang J, *et al.* 2011). Once the checkpoints are activated, they delay the cell cycle machinery until the flaw can be fixed or the danger of a mutation has been avoided (Elledge SJ. 1996; Wang J, *et al.* 2011).

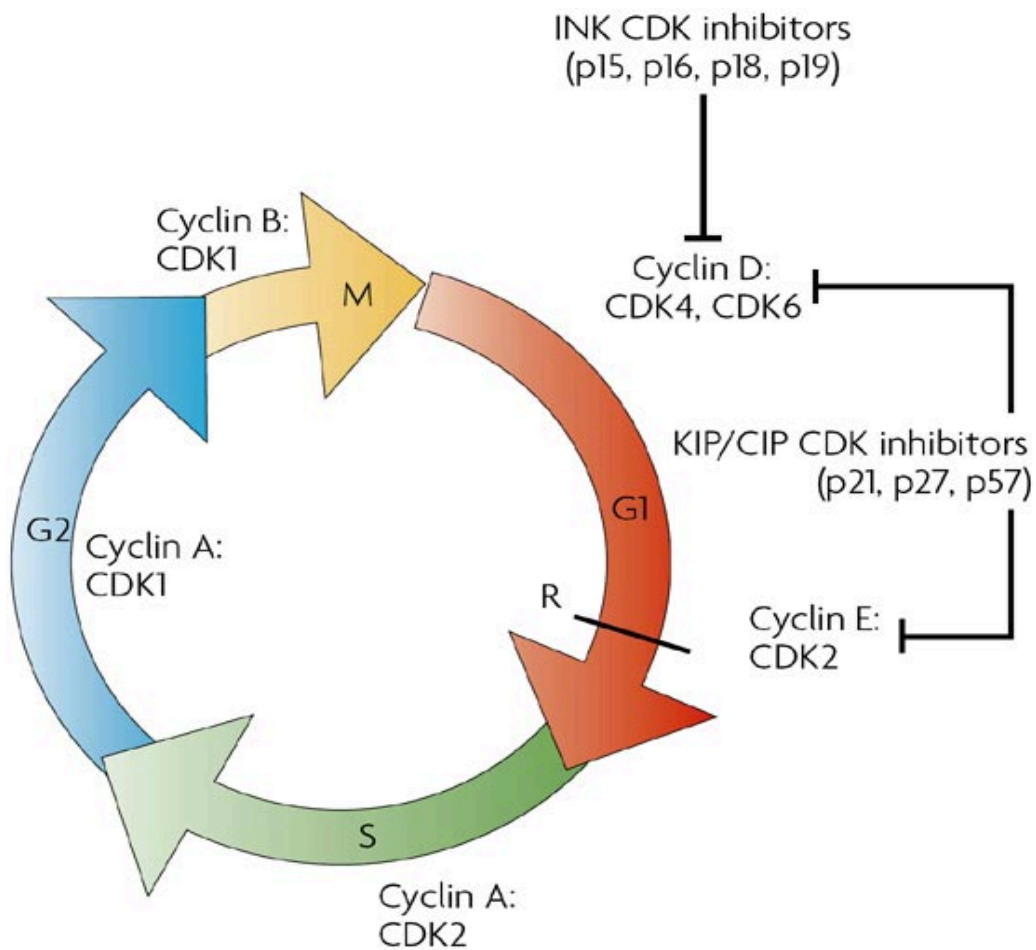


Figure 1.4: Cell Cycle of eukaryotic cells. Different phases of cell cycle include G1 (Gap 1 phase), G2 (Gap 2 phase), S (DNA synthesis phase), and M (mitosis phase). Two checkpoints are at G1/S and G2/M, which prevent damaged cells from entering synthesis phase and mitosis phase. (Dehay C and Kennedy H. *Nat Rev Neuroscience* 8. 2007)

The G1/S checkpoint prevents cells from entering the S-phase by inhibiting initiation of replication in the presence of DNA damage (Wang J, *et al.* 2011). Similarly, the G2/M checkpoint prevents cells from undergoing mitosis in the presence of damaged DNA (Wang J, *et al.* 2011). Studies by Gadbois DM, *et al.* (1996) showed an increase in p53 and p21 levels in cells shortly after radiation and suggested that G1 phase delay is dependent on the presence of p53 since DNA damage leads to accumulation of p53, which up-regulates the p21/cip-1 protein; high levels of p21/cip-1 bind to the cyclin/CDK complex and inhibit its activity. Since the cyclin/CDK complex is an important coordinator of cell cycle progression, its inactivation results in cell cycle delay (Gadbois DM, *et al.* 1996).

1.4.3 Autophagy

Autophagy is a catabolic process in which cells break down their own internal components to release energy when under different forms of stress. Autophagy means 'self-eating'. Autophagy is generally required to maintain basal level cellular and tissue homeostasis and is an essential response under conditions of stress such as nutrient deprivation, radiation, exercise and infections (Choi AM, *et al.* 2013a). During stress such as nutrient deprivation, autophagy breaks down existing cellular components to produce energy and to degrade organelles being damaged by reactive oxygen species production. It also plays an important role in the adaptive immune response such as lymphocyte development and antigen presentation (Levine B, *et al.* 2011).

A typical autophagy (macroautophagy) process is highly conservative and starts with formation of a membrane known as the phagophore or elongation membrane, which goes on to entrap various cellular components and organelles (Mizushima N. 2007). Autophagosomal elongation is regulated by two ubiquitin-like conjugation systems, the ATG5-ATG12 pathway and ubiquitin-like protein microtubule-associated protein 1 light chain 3 (LC3, ATG8) conjugation system (Honscheid P, et al. 2014). After lipidation of LC3II with phosphophtidylethanolamine, the autophagosome membrane elongates. Complete sequestration of these cellular bodies leads to formation of a double-membrane vesicle known as the autophagosome.

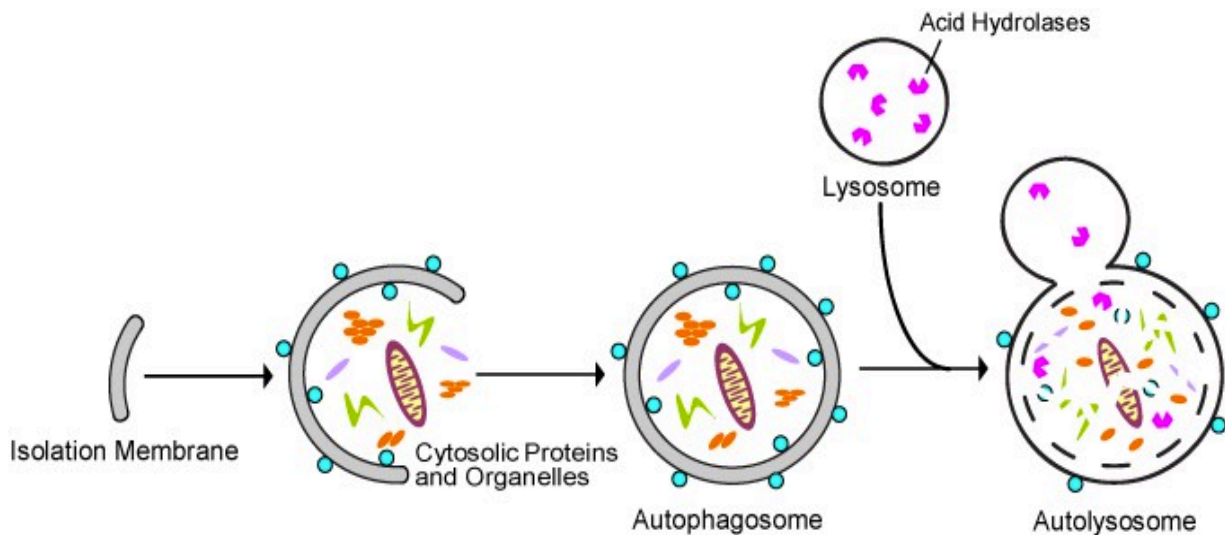


Figure 1.5. Different stages of the autophagic process. The process of autophagy starts with formation of an isolation membrane, which further elongates to incorporate various cellular components and leads to formation of a double membrane structure, autophagosome, which further fuses with lysosomes to form autolysosome, where degradation of cellular components takes place. (Watson JP and Giuliano V. 2013)

Once the autophagosome is formed, it fuses with lysosomes to form autolysosomes, where various hydrolase enzymes degrade the cellular components and organelles as shown in figure 1.5 (Mizushima N. 2007). Autophagolysosomes are single membrane structures since their inner membrane has been degraded by lysosomal/ vacuolar hydrolases (Mizushima N. 2007).

Different types of autophagy are: Macroautophagy, Microautophagy and Chaperone-mediated autophagy (as shown in the figure 1.6) (Honscheid P, *et al.* 2014). Macroautophagy (as explained above) is a process where cellular components are engulfed in double membrane structures known as autophagosomes. So far this is the best-characterized form of autophagy (Kaplan K, *et al.* 2008; Choi AM, *et al.* 2013a). In contrast to macroautophagy, for microautophagy no intermediate vesicles (autophagosomes) are required and cellular components are directly engulfed at the lysosomal surface (Honscheid P, *et al.* 2014). In this type of autophagy the lysosomal vacuole membrane either invaginates or extends via arm-like protrusions to engulf portions of cytoplasm (Uttenweiler A, *et al.* 2005; Muller O, *et al.* 2000). In microautophagy, the amino acid glutamine is a key nutrient-signaling molecule that signals and activates the mTOR pathway that ordinarily maintains inhibition of autophagy. Glutamine starvation leads to mTOR inactivation, by localization and activation of transcription factors Rtg1p/Rtg3p acting upstream of mTOR (Crespo JL, *et al.* 2002). Downstream executers of mTOR involve a number of autophagy related

genes and proteins such as ATG5, Beclin-1, ATG7, ATG8, ATG12, which are implicated in cell physiology and pathology (Liu B, *et al.* 2010).

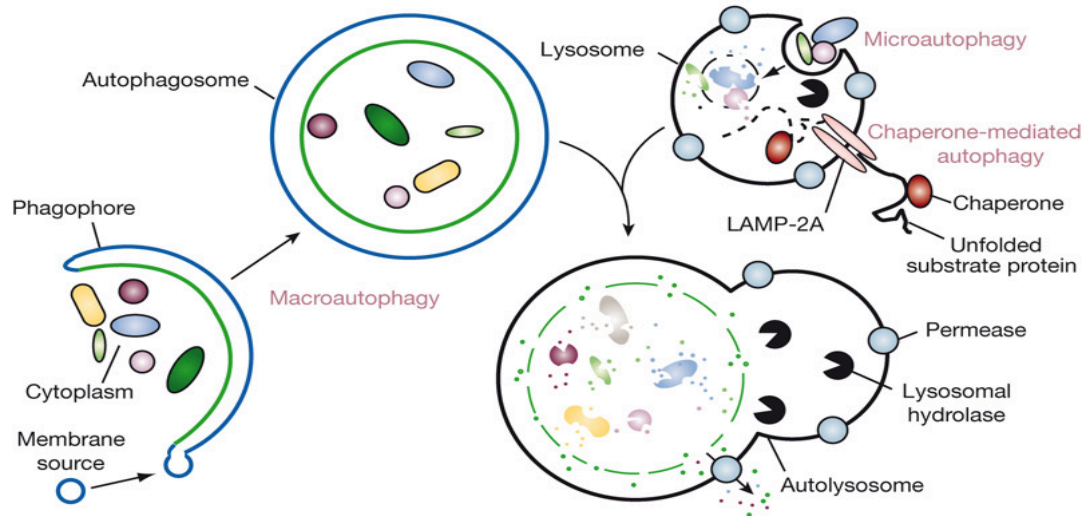


Figure 1.6. Different types of Autophagy. Three different types of autophagy are Macroautophagy, Microautophagy and chaperone-mediated autophagy. (Mizushima N *et al.* Nature. 2008)

In chaperone-mediated autophagy, a cytosolic chaperone protein known as Heat shock cognate protein 70 (HSC70), binds to substrates with specific sequences and is recognized by lysosomal associated membrane protein 2A (LAMP2A). After binding to LAMP2, the protein substrate unfolds and is translocated across the lysosomal membrane (Honscheid P, *et al.* 2014). Similar to microautophagy, chaperone mediated autophagy does not involve double membrane autophagosome formation. Studies conducted by Wang Y, *et al.* (2008) with murine embryonic fibroblasts (MEFs) from *atg5* null mice showed increased resistance to mendiaone-induced toxicity (Wang Y, *et al.* 2008). They further indicated that the protection against death from mendiaone was mediated by up-

regulation of chaperone-mediated autophagy (CMA), which occurred in atg5-/- MEFs in compensation for the loss of macroautophagy (Wang Y, *et al.* 2008). An increase in chaperone-mediated autophagy was indicated by an increase in intracellular distribution of LAMP-2A/hsc70 enriched lysosomes (Wang Y, *et al.* 2010).

Autophagic Flux. The processing of the organelles and cellular components being degraded once the autophagosome fuses with the lysosome is known as autophagic flux. The p62 protein is often used as an autophagic flux marker. p62 binds to LC3II, is transported into the autophagosome and is degraded when the autolysosome is formed. p62 levels are inversely correlated to autophagy levels.

Autophagy's contributions to cancer are contradictory. Autophagy can participate in the elimination of reactive oxygen species produced by mitochondria and other aberrant proteins aggregates, and thereby play an anti-carcinogenesis role (Scherz-Shouval R *et al.* 2007). It is thought that low levels of ROS plays a role in cell signaling, but at higher levels, redox active proteins can amplify oxidative damage and cause damage to mitochondrial (Ballinger SW, *et al.* 2000). Scherz-Shouval R *et al.* (2007) assessed the role of ROS in autophagy induction by using two fluorescent probes dihydroethidium (DHE) and 2',7'-dichlorofluorescein diacetate (DCF-DA). DHE reacts with peroxides and DCF-DA reacts with H₂O₂ to form compounds that upon binding to DNA fluoresce and can be visualized by confocal microscopy. Their studies indicate that autophagy is essential for removal of defective organelles generating uncontrolled ROS (Scherz-Shouval R,

et al. 2007). Additionally, studies done by Kirkland RA, *et al.* (2002) showed an increase in H₂O₂ and autophagosome accumulation upon starvation. Furthermore, NAC (N-acetyl cysteine), which is a ROS scavenger led to a decrease in autophagy, implicating ROS signaling as an important regulator of autophagy (Kirkland RA, *et al.* 2002).

Autophagy can also enhance tumor growth by conferring resistance against metabolic stress (Wang RC, *et al.* 2012). This can be achieved by recycling proteins and organelles in already existing tumors. Cell viability studies done by Degenhardt K *et al.* (2006) showed that inhibiting autophagy by silencing Beclin-1 or atg5 impaired survival in immortalized mouse epithelial cells, consistent with the protective nature of autophagy (Degenhardt K *et al.* 2006).

1.5 Multiple forms/functions of autophagy

Given the contradictory role(s) of autophagy in cancer development and tumor maintenance, before deciding on a potential course of treatment, it is necessary to understand what functional role autophagy is playing so that it can be exploited for therapeutic advantage. It has been long known that autophagy produced in response to radiation/chemotherapy can be protective in nature since inhibiting autophagy often leads to increased sensitivity of cancer cells to different treatment modalities (Murrow L, *et al.* 2013; Wilson EN, *et al.* 2011; Bristol ML, *et al.* 2012). Therefore, it has been anticipated that interfering with what appears to be a uniform response to stressors such as radiation and chemotherapy could provide a new direction in the enhancement of anticancer

treatment (Yang ZJ, *et al.* 2011; Sui X, *et al.* 2013). A number of clinical trials are ongoing using chloroquine/hydroxychloroquine, which is the only FDA approved autophagy inhibitor, in combination with various conventional treatments in order to enhance their effectiveness (Sperber K, *et al.* 1997; Tsakonas E, *et al.* 2000). Although a positive result from these trials would be ideal, there are a number of caveats that may have been overlooked before initiating these trials (Gewirtz DA. 2014). First, it is not possible to predict or determine whether radiation or the drugs being used are actually generating the cytoprotective form of autophagy.. Second, there is a possibility that autophagy is not a uniform/consistent response to radiation and chemotherapy. As described below, there are at least three additional forms/functions of autophagy in addition to the protective function. However, there is no biochemical or molecular way of distinguishing between these different forms of autophagy physiologically or clinically. Third, we do not have any reliable clinical marker for identifying and quantifying autophagy induction in patients' tumors. Fourth, given the pharmacokinetics of chloroquine/hydroxychloroquine, it is uncertain as to whether these drugs can achieve concentrations in tumors sufficient enough to effectively block autophagy. Fifth, since it is known that autophagy is required for routine elimination of damaged proteins, autophagy deficiency is associated with human diseases (Sarkar S. 2013). Any drug that inhibits autophagy in tumors will likely have the same impact on normal cells as well. Since chloroquine/hydroxychloroquine are given systematically and not specifically to the cancer, it is likely to be detrimental to normal tissue homeostasis.

Different forms of autophagy that have been identified in our laboratory are as follows:

1.5.1. Cytoprotective autophagy

As stated earlier, there is no way to differentiate between different functions of autophagy based on morphology or the biochemical or molecular impact of the different forms. The protective function is defined empirically essentially because blocking it either by using pharmacological inhibitors or genetic silencing leads to increased sensitivity of tumors to the autophagy-inducing stress, such as radiation (Murrow L, *et al.* 2013; Wilson EN, *et al.* 2011; Bristol ML, *et al.* 2012). Most commonly, this sensitization is via promotion of apoptosis Elmore S, 2007; Ko A, *et al.* 2014). However, it is important to show that blocking autophagy actually leads to sensitization by performing clonogenic survival or cell viability studies. The idea of cytoprotective autophagy is based on the premise that induction of autophagy confers resistance to the inducing agent and that inhibiting this form would enhance treatment response. However, selectivity still remains a problem with using autophagy inhibitors. Since defective autophagy is associated with a number of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Sarkar S. 2013), autophagy inhibition in normal cells can be detrimental. Furthermore, drugs and treatments that lead to induction of protective autophagy in tumor cells would do the same even in normal cells causing an increased drug toxicity when autophagy is inhibited. This issue of cell toxicity could be short term in situations where autophagy inhibition

occurs for a very brief time period and normal cells can recover faster than the tumor cells.

In a seminal study performed by the Kroemer group, the putative cytotoxic function of autophagy for conventional antitumor drugs was largely put to rest (Shen S, *et al.* 2011). Their studies demonstrated that inhibiting autophagy by knocking down ATG7 did not result in any protection from their anti-proliferative or cytotoxic actions. This finding if extrapolated to clinics, would support the potential utility of autophagy inhibition as a therapeutic strategy if, in fact, conventional drugs promote solely cytoprotective autophagy.

1.5.2. Non-protective autophagy

In this form, a number of stress inducers promote autophagy, but inhibiting autophagy does not alter sensitivity to the stressor. Studies performed in our laboratory have shown that in breast tumor cells (Hs578t) (Chakradeo S, *et al.*- in preparation) and murine breast tumor cells (4T1s) (Bristol ML, *et al.* 2013), inhibiting autophagy with chloroquine did not alter sensitivity of tumor cells to radiation. One of the possible explanations for lack of sensitization altering effects *in vivo* (Bristol ML *et al.*, 2013) could be related to Kroemer's findings about how autophagy inhibition interferes with the immune system's capability to recognize tumors undergoing a stress response (Michaud M, *et al.* 2011; Ko A *et al.* 2014). These studies indicate that besides other concerns, it is imperative to determine that a particular treatment is generating cytoprotective form of autophagy before considering making efforts to inhibit it as a treatment strategy.

1.5.3. Cytotoxic autophagy

Previous studies from our laboratory have shown that vitamin D and its analogs when used in combination with IR are able to promote autophagy that is cytotoxic in nature (Bristol ML, *et al.* 2012; Wilson EN, *et al.* 2012). Other laboratories have also reported generation of a cytotoxic form of autophagy by either killing the cells itself or by acting as a precursor to apoptosis (Sui X, *et al.* 2013). In the cytotoxic form of autophagy, there is a reduction in viable cell number as well as a reduction in clonogenic survival upon treatment. However, fundamentally it is differentiated from the cytoprotective autophagy in that when cytoprotective autophagy is inhibited, cells are sensitized to the treatment modality whereas when cytotoxic autophagy is inhibited, cells become less sensitive to the treatment.

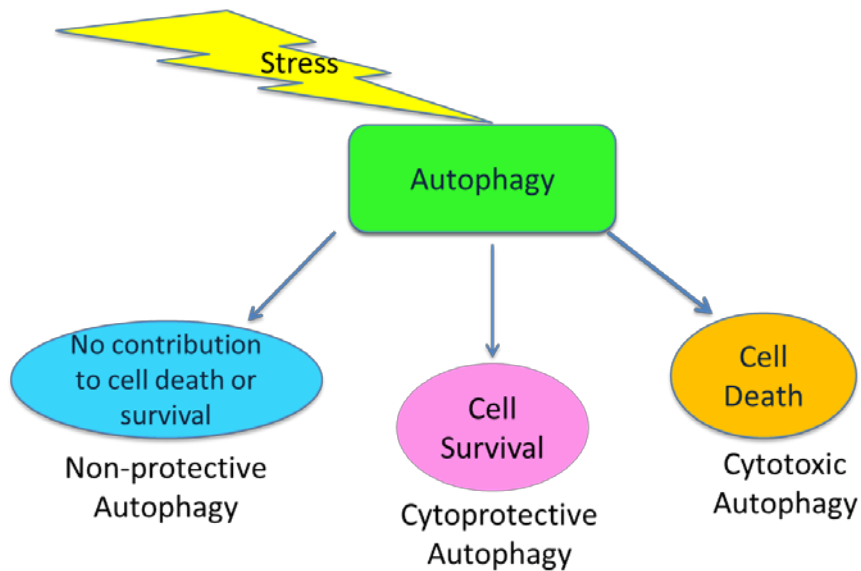


Figure 1.7 Different functions of autophagy induced in response to different stresses and cell type. Non-protective autophagy; does not seem to contribute to cell death or survival. Cytoprotective autophagy; it contributes to cell survival. Cytotoxic autophagy; induces cell death in response to the treatment.

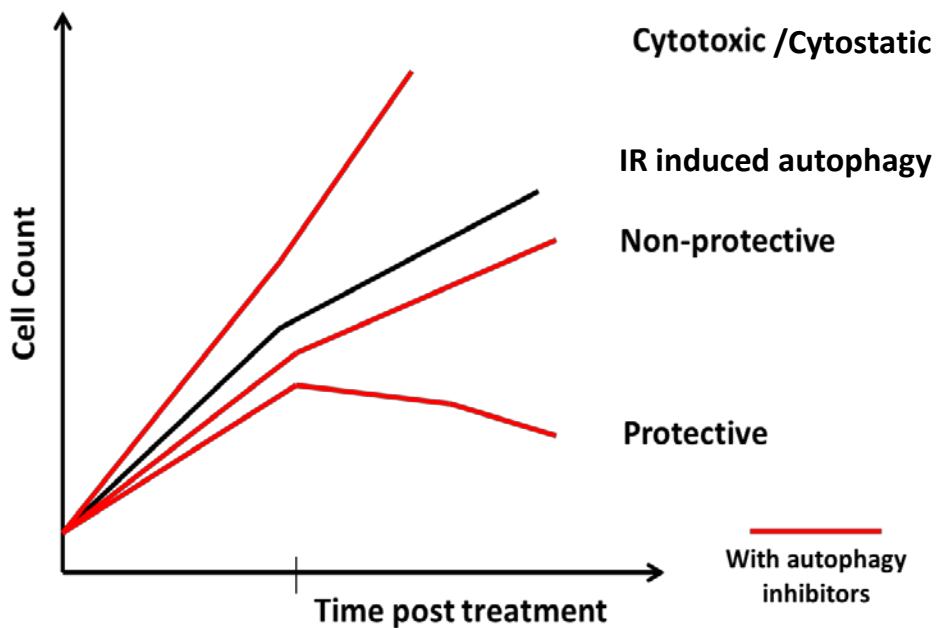


Figure 1.8 Comprehension of different functions of autophagy upon autophagy inhibition. Cytotoxic/cytostatic function; autophagy inhibition leads to a reversal in sensitization. Non-protective autophagy; Inhibition of autophagy does not alter sensitization. Cytoprotective autophagy; Inhibition of autophagy sensitizes the cells to treatment.

1.6 Signaling pathways associated with autophagy

1.6.1 AMPK/ULK-1 pathway

AMPK, a widely recognized sensor of cellular energy status, phosphorylates a number of target proteins with energy metabolism related functions in response to an ATP-depleted adenine nucleotide pool (Roach PJ. 2011). A number of studies have linked activation of AMPK to increased autophagic activity (Samari HR, *et al.* 1998; Wang Z, *et al.* 2001). Typically, AMPK exists as a heterodimer consisting of one catalytic subunit, α , and two regulatory subunits, β and γ . When intracellular levels of ATP are low, ADP or AMP can bind directly to γ subunits, which leads to a conformational change that causes phosphorylation of the catalytic subunit, α , leading to activation of AMPK (Xiao B, *et al.* 2011; Oakhill JS, *et al.* 2011). Another possibility by which AMPK can be activated is direct phosphorylation at Thr 172 by a serine/threonine kinase LKB1, which is a tumor suppressor gene (Hawley SA, *et al.* 2003; Woods A, *et al.* 2003; Shaw RJ, *et al.* 2004). AMPK can also be activated by phosphorylation at Thr172 by CAMKK β in response to alterations in calcium flux (Hurley RL, *et al.* 2005; Woods A, *et al.* 2005; Fogarty S, *et al.* 2010). Interestingly, some studies further indicate activation of AMPK by the tumor suppressor gene, p53 (Alexander A *et al.* 2011; Feng Z *et al.* 2005).

In response to nutrient starvation, AMPK acts as a regulator of cell growth. One of the best-described mechanism by which it modulates cell growth is by

inhibiting the mammalian target of rapamycin complex 1 (mTORC1) pathway (Mihaylova MM, *et al.* 2011). Studies have linked AMPK to mTORC1 control by two separate pathways (Wullschleger S, *et al.* 2006). One, AMPK inhibits mTORC1 by phosphorylating TSC2 and deactivating Rheb GTPase (Inoki K, *et al.* 2003) and second, phosphorylation of Raptor component of mTORC1 with subsequent recruitment of 14-3-3 proteins lead to inhibition of mTOR activity (Gwinn DM, *et al.* 2008).

Among various components, which regulate autophagy, mTORC1, which is a serine/threonine protein kinase, remains an important component that regulates the balance between growth and autophagy in response to environmental and physiological stress (Jung CH, *et al.* 2010). mTORC1 acts as a sensor of cellular nutritional starvation, stress and growth factor signaling. mTORC1 activity is inhibited under nutrient deprivation, which leads to autophagy induction in eukaryotes (Noda T, *et al.* 1998; Scott RC, *et al.* 2004). mTORC1 has a broad range of cellular functions; it is involved in regulation of translation, transcription and metabolism in response to growth factors in yeast and higher eukaryotes (Jung CH, *et al.* 2010). This further makes this protein kinase an important element to study for cancer, neurodegenerative diseases, metabolism and aging (Jung CH, *et al.* 2010).

In order to fully understand how mTORC1 is able to regulate the autophagy machinery, it is important to study mechanisms downstream of mTORC1. In yeast, Atg1 is the first signaling component downstream of mTORC1 in the autophagy pathway, which plays a key role in the initiation stages of autophagy

such as nucleation and formation of preautophagosome structures (PAS) (Noda T, *et al.* 1998; Straub M, *et al.* 1997; Matsuura A, *et al.* 1997). Interaction of Atg1 with other autophagy proteins such as Atg13, Atg17, Atg29, and Atg31 depends on the nutrient status and mTORC1 activity (Kamada Y, *et al.* 2000; Funakoshi T, *et al.* 1997; Kawamata T, *et al.* 2008; Kabeya Y, *et al.* 2009, 2005). mTORC1 inhibits autophagy by phosphorylating Atg13 at multiple sites to prevent its binding to Atg1. However, under starvation mTORC1 is inactivated and phosphorylation of Atg13 is suppressed. This then results in binding of Atg1 to Atg13 and other essential autophagy proteins to induce autophagy.

The counterpart of Atg1 in mammalian cells is ULK (unc-51 like kinase). As per recent literature, interaction of ULK1 and ULK2 with the mammalian homologue of Atg13 plays an essential role in autophagosome formation (Jung CH, *et al.* 2003; Chan EY, *et al.* 2009; Chang YY, *et al.* 2009; Ganley IG, *et al.* 2009; Hosokawa N, *et al.* 2009a,b). Other proteins such as Atg101 and FIP200 (focal adhesion kinase family interacting protein) interact with ULK1 and to promote autophagosome formation in mammals (Hara T, *et al.* 2008; Mercer CA, *et al.* 2009; Hosokawa N, *et al.* 2009). mTORC has been shown to regulate kinase activities of ULK1 and ULK2 (Hara T, *et al.* 2008; Jung CH, *et al.* 2003; Ganley IG, *et al.* 2009; Hosokawa N, *et al.* 2009). Conditions such as leucine deprivation or rapamycin treatment, which lead to inhibition of mTORC1, have been shown to enhance kinase activities of ULK1/2 and trigger phosphorylation of Atg13 and FIP200 (Jung C, *et al.* 2003, Ganley IG, *et al.* 2009, Hosokawa N, *et al.* 2009). Current literature suggests that the Atg1/ULK and mTORC1 complexes play a

key role in the pathways that regulate growth and autophagy in response to various pathophysiological and nutritional conditions. Atg1/ULK negatively regulate mTORC and growth to ensure favorable conditions for autophagy induction. Recent studies further link AMPK and ULK1 in mammalian cells (Egan DF, *et al.* 2011; Kim J, *et al.* 2011; Lee JW, *et al.* 2010). These studies indicate that nutritional deprivation activates mammalian AMPK, which further leads to phosphorylation and activation of ULK-1 (Roach PJ. 2011).

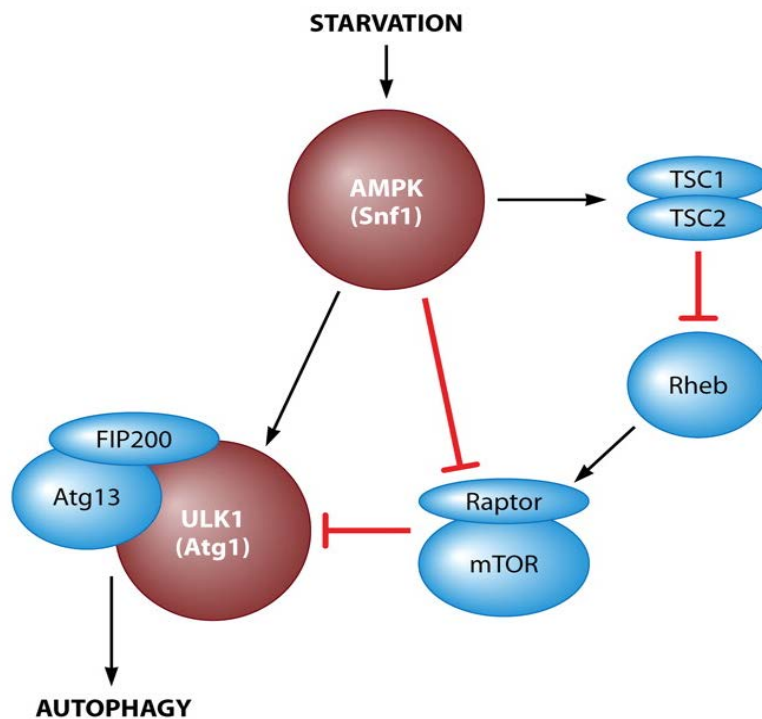


Figure 1.9 Role of AMP-activated protein kinase in the control of autophagy. AMPK when activated leads to induction of autophagy either by activating ULK-1 or by inhibiting mTOR. Roach PJ. 2011

1.6.2 p53 and its role in autophagy

The p53 tumor suppressor gene is known to induce senescence and apoptosis (Levine AJ *et al.* 2006). Its pro-apoptotic function is carried out at two levels; one is through activating pro-apoptotic proteins such as Noxa, Bax and Puma and the other one is independent of its nuclear function where its physical interaction with multi-domain members of the Bcl-2 family at mitochondria leads to mitochondrial membrane permeabilization (Zong WX, *et al.* 2008). The pro-senescence function of p53 is mediated by activating cell cycle inhibitors (Zong WX, *et al.* 2008). Recent literature suggests that in addition to its tumor suppressor function, p53 is also involved in regulation of autophagy (Zong WX, *et al.* 2008; Levine B, *et al.* 2008).

One possible mechanism for autophagy initiated by p53 could be activation of AMPK kinase, which subsequently activates TSC1 and TSC2 kinases to cause mTOR inhibition (Inoki K, *et al.* 2003)). Another possibility is activation of DRAM (damage-regulated autophagy modulator), which is a lysosomal protein and a target of p53 (Zhang XD, *et al.* 2013). Studies conducted by Zhang XD *et al.* further established DRAM1's role in regulation of autophagic flux by using A549 cells, which have wild-type TP53. An increase in autophagic flux was observed on using a mitochondrial inhibitor, 3-nitropropionic acid (3-NP), indicated by an increase in protein levels of DRAM1 and LC3II and a decrease in p62 protein. Furthermore, knockdown of TP53 in cells impaired DRAM induction, indicating TP53's involvement in DRAM mediated autophagy (Zhang XD, *et al.* 2013).

p53 appears to be capable of playing a dual role and has autophagy inhibiting and/or inducing functions (Zong WX, *et al.* 2008; Tasdemir E, *et al.* 2008a,b; Morselli E, *et al.* 2008). An increase in DNA damage, chromosomal instability and deregulated control of cell growth is caused by loss of autophagy genes, indicating a potential overlap in p53 actions and tumor suppressor-related autophagy effects (Levine B, *et al.* 2008). When cells are exposed to genotoxic stress, activation of p53 leads to stimulation of autophagy (Feng Z, *et al.* 2005; Zeng X, *et al.* 2007; Abida WM, *et al.* 2008). In contrast, p53 loss induces autophagy in the absence of stress, suggesting that basal levels of p53 activity could be inhibiting autophagy (Tasdemir E, *et al.* 2008a). Tasdemir *et al.* performed studies using HCT116 cells and used TEM to indicate increases in autophagy upon inhibition of p53 using both genetic and pharmacological inhibition approaches (Tasdemir E, *et al.* 2008a). p53 seems to be playing a central role in the process of autophagy, such that both its activation and suppression can lead to autophagy in response to wide variety of cell damage signals (Zong WX, *et al.* 2008).

The precise role of p53 has been suggested to be dependent on its localization within the cell (Chaachouay H, *et al.* 2011). ATM signaling in the nucleus appears to play an important role in radiation-induced autophagy. Nuclear p53 undergoes post-translational activation by ATM following exposure to IR (Pan J *et al.* 2009). Once activated, p53 can transactivate a number of genes that upregulate autophagy (Alexander A, *et al.* 2010; Feng Z *et al.* 2005). To understand how p53 inhibits autophagy, Tasdemir E, *et al.* (2008a) compared the

transcriptome of wild type and p53^{-/-} HCT116 cells but failed to find any changes in autophagy-related transcripts suggesting that p53 suppression of autophagy could be transcription independent (Tasdemir E, *et al.* 2008a). To investigate this further, HeLa cytoplasm (enucleated cells) were used, which in response to PFT- α (p53 inhibitor) still led to an increase in GFP-LC3 puncta, indicating that nuclei were not required for PFT- α -stimulated autophagy (Tasdemir E, *et al.* 2008a). To understand the mechanism by which p53 inhibits autophagy further experiments were conducted using p53^{-/-} HCT116 cells, which were transfected with p53 targeted to either nuclear or extra-nuclear locations. Both wild-type and endoplasmic reticulum (ER)-targeted p53 led to the inhibition of autophagy as compared to the p53 form locked into the nucleus (trapped in the nucleus using a disrupted nuclear export signal) as indicated by GFP-LC3 immunofluorescence staining (Tasdemir E, *et al.* 2008a). These studies suggest that cytoplasmic, not nuclear, p53 is required for inhibition of autophagy.

Given the numerous roles that p53 can play and the processes in which it is involved, genome wide studies have been conducted to study its transcriptional responses. An early study in 2006 by Wei *et al.* made use of chromatin immunoprecipitation (ChIP) paired end ditag (ChIP-PET) technology and defined a number of novel p53 target genes whose expression levels and functions correlated with clinical outcomes in cancer patients (Wei CL, *et al.* 2006). Since then a number of studies using ChIP technology have been used to gain novel insights into p53 biology (Lee KH, *et al.* 2010; Li M, *et al.* 2012). Recently a study conducted by Broz *et al.* identified a host of autophagy genes that are being

induced by p53 and its family members p63, p73. Their studies suggested that autophagy network activation is a fundamental element of p53 biological function (Kenzelmann Broz D, *et al.* 2013) and highlighted the importance of the transcriptional activity of p53 for the induction of autophagy. Furthermore, they indicated that deficiency/inhibition of autophagy did not alter DNA damage-induced cell cycle arrest and/or survival but induced p53-dependent apoptosis. However, the question of how exactly autophagy contributes to p53 dependent apoptosis is still not answerable (Kenzelmann Broz D, *et al.* 2013).

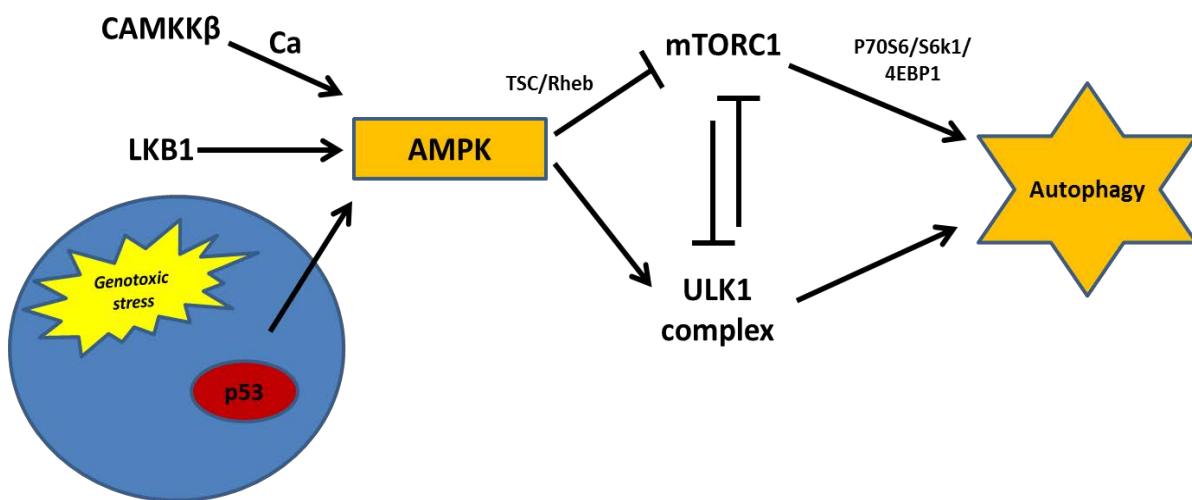


Figure 1.10. Proposed model for autophagy signaling pathway in response to radiation in NSCLC. Genotoxic stress (such as IR+EB 1089) leads to stabilization and activation p53, which leads to activation/phosphorylation of AMPK. AMPK further induces autophagy by either activation of ULK-1 or inhibition of mTOR.

1.7. Vitamin D

Vitamin D is a hormone known for its role in calcium and phosphorus homeostasis. The majority of vitamin D is synthesized in the body by exposure to sunlight (Norton R, *et al.* 2012). Moderate amounts can also be obtained from diet such as fish, and fish-oils (Norton R, *et al.* 2012). Cholecalciferol, a precursor of vitamin D₃, is obtained by UVB radiation's action on 7-dehydrocholesterol in the skin (Norton R, *et al.* 2012). Vitamin D precursors are then taken to the liver by vitamin D binding proteins (VDBP), where they are hydroxylated to be converted to 25-dihydroxyvitamin D (25(OH)D or calcidiol). 25(OH)D is the form in which vitamin D circulates in the body and is used as a measurement of vitamin D status in an individual (Norton R, *et al.* 2012). 25(OH)D is further hydroxylated by the cytochrome 1 α -hydroxylase enzyme CYP27B1 in the kidneys to produce the most potent and biologically active form 1,25-dihydroxyvitamin D (1,25(OH)₂D or calcitriol), which has a half-life of 15hr (Radermacher J, *et al.* 2006; Hansdottir S, *et al.* 2008; Shaffer PL, *et al.* 2002). Another cytochrome 24-hydroxylase CYP24A1 enzyme further metabolizes both 25(OH)D and 1,25(OH)₂D to 24,25-dihydroxycholecalciferol (24,25(OH)₂D) to keep vitamin D concentrations in blood strictly regulated (Holick MF *et al.* 2007). 1,25(OH)₂D₃, the active form of vitamin D is able to mediate its effect only after binding to the vitamin D receptor (VDR) (DeLuca HF. 2004).

The human VDR protein consists of 427 amino acids and has a DNA-binding domain, a ligand binding domain and an activating domain (DeLuca HF. 2004).

$1,25(\text{OH})_2\text{D}_3$ binds to the ligand binding domain located at the carboxyl terminus of the VDR protein. This binding changes VDR's conformation, allowing interaction with transcription factors (Shaffer PL, *et al.* 2002). Once activated, VDR binds to retinoic acid X receptor and forms a heterodimer.

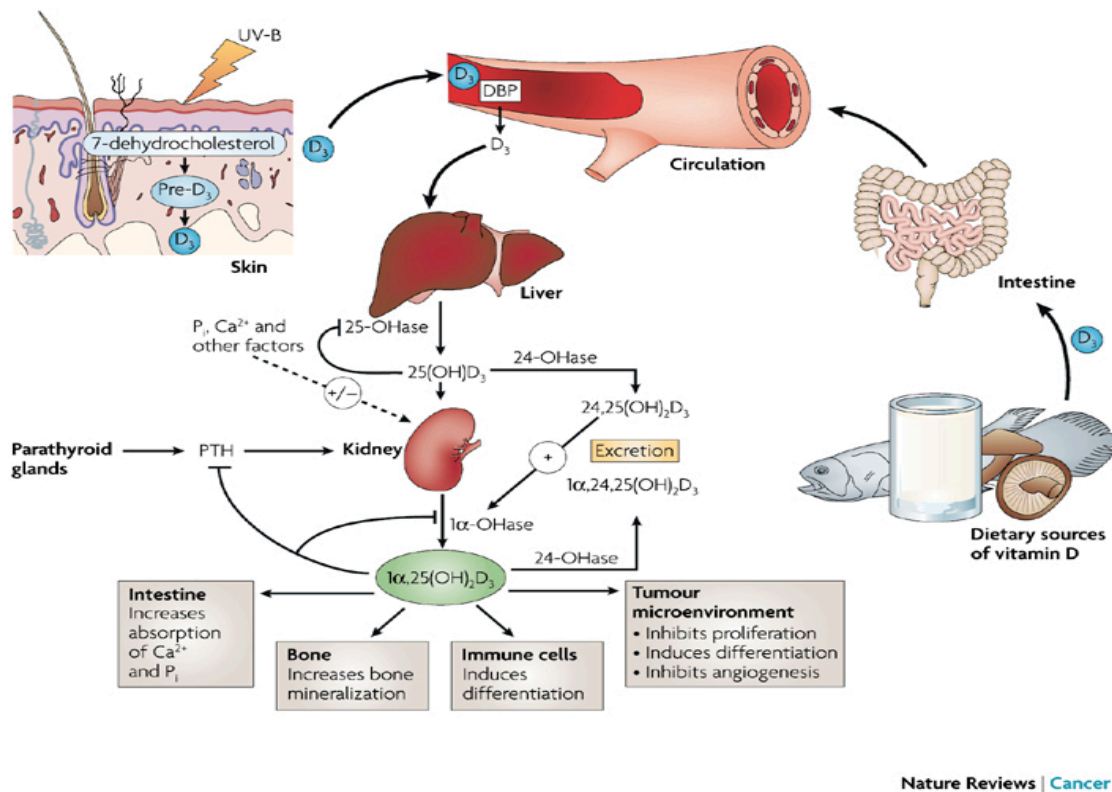


Figure 1.11. Vitamin D metabolism. Vitamin D obtained from either diet or sun, has to undergo two hydroxylations to be converted into its active form, $1,25(\text{OH})_2\text{D}_3$. (Deeb KK *et al.* *Nature Reviews Cancer* 7. 2007)

This heterodimer then translocates to the nucleus, where it binds to the vitamin D response elements in the promoter region of target genes (Nagpal S, *et al.* 2005). Therefore, most of the effects of vitamin D are VDR dependent (Holick

MF. 2007). However, studies have indicated that vitamin D can also have some rapid non-genomic effects such as rapid regulation of membrane calcium channels in cultured chick skeletal muscle cells (Vazquez G, *et al.* 1997). These rapid responses are mediated by a new receptor known as 1,25-D₃-membrane-associated-rapid-response-steroid-binding-protein-receptor (1,25MARRS) (Nemere I, *et al.* 1998,2004). Studies in MCF-7 breast tumor cells indicate that the presence of this receptor interferes with the anti-proliferative effects of 1,25(OH)₂D₃ (Richard CL, *et al.* 2010; Wu W, *et al.* 2010). Further investigation needs to be done to determine the exact role of this receptor in anti-proliferative effects of 1,25(OH)₂D₃.

1.7.1 Vitamin D and autophagy

Vitamin D leads to increased intestinal calcium absorption by activating VDR (Song Y, *et al.* 2003). ER releases this calcium, which activates calcium-dependent kinases and phosphatases and leads to activation of various cellular processes, including autophagy (Hoyer-Hansen, *et al.* 2007). Some of the signaling pathways that vitamin D₃ regulates include Beclin-1, mammalian target of rapamycin (mTOR), the class III phosphatidylinositol 3-kinase complex (PI3KC3) and Bcl-2 pathways. (Wu S, *et al.* 2011; Van Leeuwen and Pols, 2005). mTOR expression can be down-regulated by vitamin D₃, thus leading to an induction in autophagy (Loewith R, *et al.* 2002; Wang J, *et al.* 2008).

Beclin-1 is one of the key elements of the PI3KC3 complex (Kang R, *et al.* 2011) and sits at the core of autophagy regulation (Sun *et al.* 2009). The PI3KC3 complex is important for localization of autophagic proteins at the pre-autophagosomal structure (Kang R, *et al.* 2011; Kihara A, *et al.* 2001). Beclin-1 is regulated by a number of factors including Bcl-2 (Pattingre S, *et al.* 2005), vitamin D3 and vitamin D analogs (Hoyer-Hansen *et al.* 2005; Wang J, *et al.* 2008). However, the mechanism vitamin D uses to increase Beclin-1 expression still isn't clear. One possible mechanism could be through Bcl-2 since Bcl-2 binds directly to beclin-1 at a BH3 domain inhibiting beclin-1 and consequently autophagy (Sinha S, and Levine B. 2008). Ylikomi *et al.* (2002) conducted studies to show that 1,25-D₃ downregulates expressions of Bcl-2 in MCF7 breast tumor cells and HL-60 leukemia cells (Ylikomi T *et al.* 2002). Studies have also shown that starvation induced autophagy levels can be increased by endogenous silencing of Bcl-2, which would possibly lead to an increase in availability of beclin-1 to be recruited to bind to PI3KC3 (Maiuri MC, *et al.* 2006; Pattingre S, *et al.* 2005; Zhang XD, *et al.* 2009).

1.7.2 Vitamin D and cancer:

Vitamin D has been shown to have anti-tumorigenic effects both in-vitro and in-vivo (Norton R, *et al.* 2012). A decrease in circulating levels of 25-hydroxyvitamin D (25(OH)D), the circulating precursor of calcitriol, has been shown to be associated with increased risk of cancer (Giovannucci E. 2008, Krishnan AV, *et al.* 2010). Vitamin D has been shown to induce apoptosis in

breast and colon cancers but can have some differential effects such as growth arrest in G1-S phase as suggested by studies in prostate cancer (James CF, *et al.* 2008). Some of the other anticancer related properties of vitamin D include its antimetastatic, anti-inflammatory and antiangiogenic effects (Vanoirbeek E, *et al.* 2011).

Vitamin D has been indicated to regulate signaling pathways which impact cellular growth and differentiation via proteins such as Ras and MAPK, cyclic AMP, protein kinase A, protein lipase A, prostaglandins, and phosphatidyl inositol 3 kinase (Deeb KK, *et al.* 2007). It is also proposed to induce cyclin dependent kinase (CDK) inhibitors or inhibit cyclin D/cdk4-6, which would lead to reduced activity of the E2F transcription factor by causing hypophosphorylation of Rb (Eelen G, *et al.* 2004). A recent study by Zhou *et al.* showed that there is a close association between improved survival in early stage lung cancer and high 1,25(OH)₂D₃ circulating levels (Zhou W, *et al.* 2005, 2007). Various preclinical studies have demonstrated anti-proliferative effects of vitamin D. Inhibition of tumor growth and metastases by 1,25(OH)₂D₃ has been shown in mouse models (Young MR, *et al.* 1995).

Vitamin D has been used in combination with other treatments in lung cancer. Studies by Zhuravel E, *et al.* (2010) showed that using vitamin D in combination with an experimental cancer vaccine in the Lewis Lung Cancer model led to a significant increase in the anti-metastatic effect of the vaccination. The drug by

itself was not potent enough to cause any reduction in tumor volume and metastasis (Zhuravel E, *et al.* 2010). Furthermore using vitamin D analogues, PRI-2202 and PRI-2205 in combination with cisplatin was more effective compared to cisplatin alone (Wietrzyk J, *et al.* 2007). Studies using chemical-induced mouse models of lung cancer in Balb/c mice which developed tumors when treated with urethane show a pronounced decrease in tumor formation when given vitamin D in their diet (Koohdani F, *et al.* 2008). All of these studies support the idea of vitamin D's possible role in lung cancer therapy.

1.8. Previous studies

One of the primary goals of our laboratory has been to identify and develop strategies to enhance sensitization to radiation. One such approach is to employ vitamin D and vitamin D analogues, which are known to have anti-tumorigenic effects, in combination with radiation. Tumor recurrence is one of the major problems that lead to inefficient treatment, since the recurred tumor is more resistant to the treatment. Our laboratory has reported on studies using vitamin D analogues, EB 1089 (seocalcitol) and ILX23-7553, in combination with Doxorubicin (Topoisomerase II poison) and radiation (Gewirtz, DA *et al.* 2000; Sundaram S, *et al.* 1999; Sundaram S, *et al.* 2003).

Previous data generated by our lab has shown that active form of vitamin D, 1,25-D3 and the vitamin D analogue EB 1089 produce a significant reduction in growth of MCF7 breast tumor cells when used in combination with radiation or Doxorubicin (Chaudhry M, *et al.* 2001, Demasters *et al.* 2006, 2004; Sundaram

S, *et al.* 1999, 2003). In vivo studies conducted using EB 1089 and MCF7 breast tumor xenografts in nude mice also indicated enhancement of radiation effects (Sundaram S, *et al.* 2003). These studies also indicated that EB1089 in combination with IR or Doxorubicin failed to generate significant amounts of apoptosis and mitotic catastrophe, which were initially thought to be the mechanism of sensitization (Demasters *et al.* 2006, 2004). Studies conducted in order to determine the mode of this radio sensitization by vitamin D indicated that autophagy could be the mode of this sensitization since there was a significant increase in autophagy with the combination treatment of IR and vitamin D (Demasters *et al.* 2006).

While our laboratory has previously established that vitamin D and its active analogues such as EB 1089 are capable of radio-sensitizing breast tumor cells, the current studies were designed to build upon and expand the sensitization studies to Non-small cell lung cancer cell models, as non-small cell lung cancer is considered to less responsive and less susceptible to the effects of radiation compared to breast cancer. Breast cancer is much more amenable and responsive to treatment, for a couple of reasons. One is that it is generally detected early in its progress through screening or self-examination and therefore can be resected surgically and/or irradiated. Furthermore, there are many chemotherapeutic strategies that are effective, both in terms of conventional drugs (paclitaxel, cyclophosphamide, doxorubicin) as well as adjunctive therapies (herceptin) and hormonal therapies (tamoxifen and aromatase inhibitors) (American Cancer Society, T. 2012). One of the major

issues with treatment of NSCLC is late diagnosis; consequently the tumors are generally quite large and lie in close proximity to normal tissues, making it difficult to either resect or treat with high doses of radiation. Therefore, there is a need to sensitize these tumor cells to moderate doses of radiation. We also assessed levels of the vitamin D receptor in different non-small cell lung cancer cell lines and found that most of the cell lines that we tested express high to moderate levels of VDR, which further indicated that they are likely to respond to the combination treatment since vitamin D mediates its actions through VDR.

Our studies were designed to test the following hypotheses:

- 1,25-D₃ and EB 1089 will radiosensitize relatively radio-resistant A549 and H460 lung tumor cells.
- Promotion of autophagy will prove to be responsible for the radio-sensitization
- The AMPK/ ULK-1 pathway will play a role in the radio-sensitization of NSCLC cells by 1,25-D₃ and EB 1089.

To test our hypotheses we developed the following aims:

Specific Aim 1: To establish the capacity of 1,25-D₃ and EB 1089 to radio-sensitizes NSCLC cells using A549 and H460 cells.

Specific Aim 2: To substantiate that autophagy is the mode of radio-sensitization in NSCLC cells treated with 1,25-D₃ or EB 1089 using both pharmacological and genetic inhibition tools

Specific Aim 3: To determine the signaling pathways that might be involved in 1,25-D₃ /EB 1089 mediated radio-sensitization.

1.9. Cytostatic autophagy

A novel form/function of autophagy has been identified in our laboratory that is the focus of this thesis. Studies in non-small cell lung cancer cells exposed to vitamin D or a vitamin D analog in combination with radiation have identified a “cytostatic” function of autophagy. As with the other forms of autophagy, the definition of this form is entirely functional and empirical. The combination of vitamin D and radiation is able to induce much more pronounced inhibition in growth of NSCLC cells compared to radiation alone, which is indicative of increased sensitization. Similar to the cytotoxic effect, when this cytostatic form of autophagy is inhibited, we see a reversal in sensitization; what differentiates cytostatic from cytotoxic autophagy is the lack of cell killing with the cytostatic form, as shown by cell viability/clonogenic survival studies done with breast tumor cells (Bristol ML, *et al.* 2012; Wilson EN, *et al.* 2011) previously and non-small cell lung cancer cells in this study. The treatment conditions were the same for both tumor models.

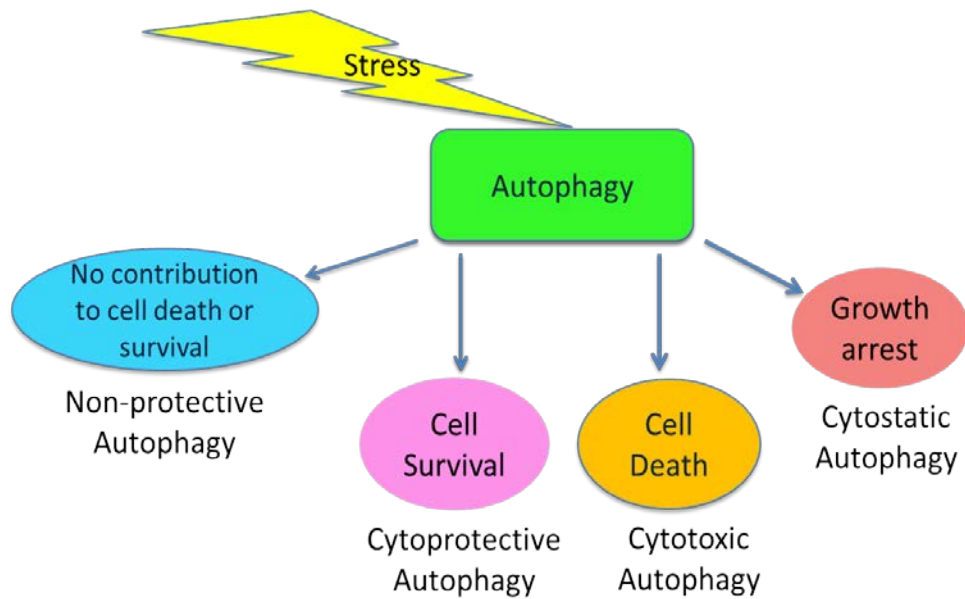


Figure 1.12 A novel function, cytostatic, identified in NSCLC cells. In addition to three other forms defined earlier, a novel form of utophagy has been identified in NSCLC cells; this function induces growth arrest in response to treatment and reverses sensitization when inhibited.

Forms of Autophagy	Characteristics
<p>Cytoprotective</p> <ul style="list-style-type: none"> Induced in p53 wild-type tumor cell lines in response to radiation treatment; A549, H460, MCF7, Zr75) 	<ul style="list-style-type: none"> a. May confer resistance to therapy b. Increased sensitivity to therapy when blocked c. Increased apoptosis when blocked d. Possibly involved in normal tissue homeostasis
<p>Cytotoxic</p> <ul style="list-style-type: none"> Induced mainly in response to the combination treatment of radiation and vitamin D in p53 wild-type breast tumor cell lines; MCF7, Zr75) 	<ul style="list-style-type: none"> a. Promotes cell death when induced b. Cell death could be associated with apoptosis c. Reversal in sensitization when blocked
<p>Cytostatic</p> <ul style="list-style-type: none"> Induced in response to the combination treatment of radiation and vitamin D in p53 wild-type non-small cell lung cancer cell lines; H460, H1299 (inducible p53)) 	<ul style="list-style-type: none"> a. Mediates growth inhibition b. Results in reduced clonogenic survival c. Potentially associated with senescence/growth arrest d. Could be involved in tumor growth delay /dormancy
<p>Nonprotective</p> <ul style="list-style-type: none"> Induced in response to radiation treatment in p53 null or mutant tumor cell lines; H1299 (p53 null NSCLC), Hs578t (p53 mutant breast tumor cell line). 	<ul style="list-style-type: none"> a. No difference in intensity when compared to other forms b. No physiological differences when compared to others c. Inhibition does not influence or alter the sensitivity to therapy d. Relevance not quite understood

Table 1: Summary of the characteristics of different functions /forms of autophagy.

Historically, from our understanding the primary function of autophagy occurring under nutrient deprivation has been to allow cell survival in a state of stasis where the metabolic state is maintained, which is essentially what we see with the cytoprotective autophagy (Levine B. 2007; Gewirtz DA. 2014). However, it must also be emphasized that this cytoprotective form is clearly different from the cytostatic form. In the same cells where we as well as Kroemer's group have demonstrated cytoprotective function of autophagy in response to radiation alone (Ko A et al. 2013), addition of vitamin D or EB1089 converts this cytoprotective function into cytostatic autophagy.

However, the therapeutic implications of cytostatic autophagy would be similar to that of the cytotoxic form. That is, if chemotherapeutic agents or radiation are promoting prolonged cell growth arrest, then suppression of autophagy is likely to reduce the impact and effectiveness of the therapy.

Chapter 2

Materials and Methods:

2.1 Cell lines:

The p53 wild-type (WT) A549, VDR null H838, and p53 mutant H358 human non-small cell lung tumor cell lines along with normal human bronchial epithelial cell line HBEC3-KT originally generated by the Minna laboratory (Sato M, et al. 2006) were generously provided by Dr. Charles Chalfant at Virginia Commonwealth University. H460 (wild-type p53), human non-small cell lung cancer cell line, was a generous gift from Dr. Richard Moran at Virginia Commonwealth University. H460 shBeclin-1, shAtg5, shcontrol cells were generated as described below. H1299 null and p53 inducible human non-small cell lung cancer cells were originally developed by Dr. Constantinos Koumenis (Maecker HL, et al. 2000) and provided by our collaborators, Drs. Frank and Suzy Torti, at the University of Connecticut.

Mission shRNA bacterial stocks for Atg5 and Beclin-1 were purchased from Sigma Aldrich (St. Louis, MO, #TRCN00151963 and #TRCN0000299864, respectively). Lentiviruses were produced in HEK 293TN cells co-transfected using Lipofectamine (Invitrogen, Carlsbad, CA) with psPAX2 and pMD2.G packaging constructs (Addgene, Cambridge, MA). Viruses shed into the media were then used to infect H460 cells. Puromycin (1µg/ml) was used as the selection marker to enrich the infected cells.

2.2 Cell culture and treatment:

H460, A549, and H1299 cells were maintained in DMEM media supplemented with 10% (v/v) FBS, 100 U/ml penicillin G sodium and 100µg/ml streptomycin sulfate. H838 cells were maintained in RPMI 1640 media supplemented with the same amounts of serum as mentioned above. HBEC3-KT cells were maintained in Keratinocyte-SFM media supplemented with FBS (given with the kit), 100 U/ml penicillin G sodium, and 100µg/ml streptomycin sulfate. H460 shAtg5 and shBeclin-1 cells were maintained with puromycin (1µg/ml) for selection. All cells were maintained at 37°C under a humidified, 5% CO₂ atmosphere, except otherwise indicated. After equilibration post seeding, cells were treated with single dose of 6Gy radiation alone (using a ¹³⁷Cs irradiator), 100nM 1,25-D₃ alone, or 100nM EB1089 (Tocris Bioscience, Bristol, UK) alone, or the combination of 1,25-D₃ or EB 1089 with 6Gy radiation dose. Cells were washed free of the 1,25-D₃ or EB 1089 after 48 hours (i.e. exposure for 24 hours prior to and 24 hours post irradiation).

2.3 Cell viability and Clonogenic survival:

Cell viability was assessed by trypan blue exclusion at various time points after the treatment. Trypan blue is a live-dead cell dye, which can enter the cells and stain them blue when they are dead and their membrane is compromised. 10,000 cells were seeded and after various treatments, cells were harvested using trypsin, stained with 0.4% trypan blue (Sigma T8154), and counted using a hemocytometer under phase contrast microscopy. For clonogenic studies, cells were plated in six well dishes at an appropriate density (specific cell numbers are mentioned in the figure legends). After 2 weeks, the cells were washed with PBS, fixed with 100% methanol, air-dried and stained with 0.1% crystal violet (Sigma C3886). Cells in groups of 50 or more were counted as colonies and data were normalized relative to untreated controls. Data is represented as number of colonies counted.

2.4 FACS Analysis for Annexin V and Propidium Iodide positive cells to assess apoptosis.

For FACS analysis, 20,000 cells were seeded and after different treatments, cells were collected and labeled fluorescently for detection of apoptotic and necrotic cells. This assay works on the following principle: In early apoptosis, phosphatidyl serine molecules are expressed on the outside of the cell membrane. Annexin V, which is fluorescently labeled with a molecule such as FITC, has great affinity for these phosphatidyl serines and binds to them. This way cells in early apoptosis give out Annexin V signal. When cells are in late apoptotic stage, their membrane starts to break

apart and PI, which is an intercalating agent and binds to nucleic acids (RNA and DNA), is able to enter the cells and now cells stain positive for both annexin V and PI. However when cells become necrotic, the cell membrane completely disintegrates and all the cellular components (including phosphatidyl serines) get degraded. Cells, when necrotic stain positive only for PI.

For sample preparation, cells were scraped off from the plate and washed twice with cold PBS. After the washes, they were re-suspended in 500 μ l of 1x Binding Buffer (FITC AnnexinV apoptosis detection kit, BD Biosciences, San Jose, CA) with 5 μ l of Annexin V-FITC, and 10 μ l of propidium iodide (50 μ g/ml). Samples were gently mixed and incubated at room temperature in the dark for 15 min. The percentage of cells with increased annexin/PI staining was assessed by flow cytometry and analyzed by BD FACSCanto II using FACSDiva software. A minimum of 10,000 cells within the gated region were analyzed. Staurosporine was used as positive control for this study. It is able to induce apoptosis by activating Caspase-3.

2.5 Detection and quantification of senescent cells.

The method described by Dimri et al. 1995, was used for detection of β -galactosidase positive cells. 20,000 cells were seeded and after treatment, cells were washed with PBS and fixed with 2 % formaldehyde/ 0.2% glutaraldehyde/PBS for 10-12 minutes. Cells were again washed with PBS and stained with 5-bromo-4-chloro-3-inolyl- β -D-galactosidase(X-Gal) in dimethylformamide (20 mg/ml), 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150

mM NaCl, and 2 mM MgCl₂ and incubated at 37°C overnight. After incubation, the cells were washed twice with PBS and visualized using an Olympus inverted microscope. All images were taken at the same magnification. Since senescent positive cells have high levels of β -galactosidase enzyme due to increased lysosomal mass, they break down X-gal to form an insoluble blue color compound. The amount of this blue color compound is an indicator of the extent of senescence.

For quantification of the senescence levels using FACS, 20,000 cells/well were seeded, treated as above and analyzed using a fluorescent β -galactosidase activity marker, C₁₂FDG (Life technologies, D2893). The protocol was adapted from Debacq-Chainiaux et al. 2009. C₁₂FDG substrate is impermeable and non-fluorescent initially but once it gets hydrolyzed by β -galactosidase enzyme, it gets trapped and gives out a green fluorescence which can be quantified using flow cytometry.

2.6 Detection and quantification of acidic vesicles with Acridine Orange staining.

Cells were plated (20,000/well) in six well culture dishes, stained with acridine orange (Sigma 10mg/ml) at a final dilution of 1:10,000 in respective culture media and allowed to incubate at 37°C for 10 min. After one wash with PBS, cells were imaged under an inverted fluorescence microscope (Olympus, Tokyo, Japan). All images were taken at the same magnification. Acridine orange is a lysotropic dye, which is a hydrophobic green colored molecule at neutral pH but when pH becomes acidic, it gets protonated and gets entrapped in the acidic vesicle and emits bright orange/red signal.

For quantification of autophagic vesicles (AVOs), 20,000 cells were seeded and after different treatments, cells were trypsinized, harvested and washed with PBS. Pellets were re-suspended in 500µl PBS, stained with a 1:10,000 dilution of acridine orange for 10 min and analyzed by BD FACSCanto II using BD FACSDiva software at the Virginia Commonwealth University Flow Cytometry Core Facility. A minimum of 10,000 cells within the gated region was analyzed.

2.7 GFP-LC3 redistribution.

GFP-LC3 plasmid was obtained from Addgene (22405). Retroviruses were produced in HEK 293TN cells transfected using Lipofectamine (Invitrogen, Carlsbad, CA). Viruses shed into the media were then used to infect H460 and A549 cells. Puromycin (1µg/ml) was used as the selection marker to enrich the infected cells. For sample preparation, cells were treated as described above, fixed with 3% paraformaldehyde, and visualized using a Leica Confocal laser-scanning microscope. Cells were counterstained with DAPI to stain and visualize the nucleus. GFP-LC3 is visualized either as a diffused cytoplasmic pool or as puncta structures that primarily represent autophagosomes.

2.8 Western blotting.

After the indicated treatments, cells were scraped from the culture dishes, collected and lysed using M-PER mammalian protein extraction reagent (Thermo Scientific, Waltham, MA) containing protease and phosphatase inhibitors. Protein concentrations were

determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Total protein was then diluted in SDS sample buffer and dry boiled for 10 min. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and blocked in 5% milk/1x PBS/ 0.1% Tween for 1 hour. Primary antibodies used at a 1:1000 dilution with overnight incubation were VDR (Santa Cruz Biotechnology), p62 (Santa Cruz Biotechnology, Santa Cruz, CA), LC3II (Cell signaling Technology), ATG5 (Cell Signaling Technology, Danvers, MA), BECN1 (Cell Signaling Technology), p53 (BD pharmingen), phospho-AMPK (Cell signaling Technology), total-AMPK (Cell signaling Technology), phospho-acetyl ACC (Cell signaling Technology), β -actin (Santa Cruz Biotechnology). The membrane was then incubated with secondary antibody of either horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:10,000; Sigma) or goat anti-mouse (1:10,000; Sigma) for 1 hour, followed by extensive washing with tween-PBS. Blots were developed using Pierce enhanced chemiluminescence reagents and Bio-Max film (Thermo Scientific).

2.9 Assessment of kinase phosphorylation and γ -H2AX formation.

20,000 cells/well were seeded and after different treatments, cells were washed twice with PBS, fixed with 4% paraformaldehyde for 10 min, permeabilized with 100% cold methanol for 30 min and incubated with blocking buffer (4% BSA in PBS) for 10 min. Cells were incubated with a 1:50 dilution of primary antibody for 1 hour, washed with PBS and incubated with a fluorochrome- conjugated secondary antibody (1:100 dilution) for 30 min in the dark. After the second incubation, cells were washed with blocking

buffer and re-suspended in 400 μ l PBS. Cells were analyzed using FACS with a minimum of 10,000 cells for each sample (Gagou EM et al. 2010). Primary antibodies used were p-AMPK α 1/ α 2 Thr172 (Cell Signaling), p-ULK-1 Ser317 (Cell signaling), and anti- γ -H2AX Ser139 (EMD Millipore, Billerica, MA).

2.10 Statistical Analysis.

Statistical differences were determined by StatView statistical software (SAS Institute, Cary, NC). The data were expressed as means \pm SE (as standard error of the mean). Comparisons were made using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test, and p values \leq 0.05 were taken as statistically significant.

Chapter 3

Sensitization to Radiation by vitamin D.

3.1 Influence of 1,25-D₃ and the vitamin D analogue EB1089 on sensitivity to radiation in non-small cell lung cancer cells

As indicated in the Introduction 1,25-D₃ and EB 1089 can influence cell function only after they bind to vitamin D receptor (VDR). Therefore, we first employed western blotting to assess VDR levels in a spectrum of non-small cell lung cancer cell lines, specifically H460, A549, H1299, H838, H358, and H2030 cells. As shown in figure 3.1, VDR was found to be expressed at high levels in A549, H358, H460 cell lines and in moderate amounts in H1299 and H520 cells while VDR expression was not expressed in the H838 and H2030 cell lines.

In order to determine a suitable dose of radiation to be used for these studies, we performed clonogenic survival studies where cells were plated at a relatively low density (mentioned in the figure legends) and were treated with different doses of radiation ranging from 2Gy to 10Gy. Results were analyzed two weeks after the treatment. As shown in figure 3.2, radiation treatment inhibited colony forming ability of the cells at all doses used.

We decided to employ 6Gy (60% inhibition of growth) in our subsequent studies in order to be able to study the sensitization/growth inhibitory effects of radiation and radiation + 1,25-D₃/EB1089 without killing the cells. As Vitamin D and its analogues have been shown to have anti proliferative effects in MCF7 breast tumor cells when used at 500 nM concentrations (Colston KW, et al. 1992), we performed clonogenic survival studies and cell viability studies using different concentrations of EB1089 and 1,25-D₃ in order to assess their effects on lung tumor cell growth. Modest growth inhibitory effects of vitamin D alone at 100nM had previously been shown in our laboratory in studies with MCF7 and ZR75 breast tumor cells (Demasters GA, et al. 2006; Wilson EN, et al. 2011). Clonogenic survival studies were carried out over a span of 2 weeks after EB1089/1,25-D₃ treatment for 24 hours. In both p53 wild-type cell lines, H460 and A549, EB1089 and 1,25-D₃ had minimal growth suppressive/inhibitory effects as shown in figure 3.3. For cell viability studies, which were performed by trypan blue exclusion, vitamin D treatment was for 24 hours and cell viability was determined on days 1, 3, 5, and 7. Similar to the clonogenic survival studies, 1,25-D₃ and EB1089 had minimal inhibitory effects on the growth of the two cell lines as shown in figure 3.4. The lack of

effect of these agents alone establishes that the sensitization effects observed are not simply the consequence of additive anti-proliferative or cytotoxic interactions.

Further, in order to establish a concentration of vitamin D that could be used in radiation sensitization studies, clonogenic survival and cell viability studies were performed using different doses of EB 1089 ranging from 5 nM to 200 nM, in combination with a single dose of 6Gy radiation. Based on studies shown in Figure 3.5, which indicate that doses above 100nM become toxic, we established that a concentration of 100 nM would be used in combination with 6Gy for our studies. This is an identical concentration to that previously reported to sensitize breast tumor cells to radiation (Milczarek M et al. 2013; Demasters GA, et al. 2006; Sundaram S, et al. 2003).

Treatment with 1,25-D₃ and EB 1089 had an essentially identical impact in terms of tumor cell sensitization. Again, this is similar to our previous findings in studies with breast tumor cells. Consequently, while the bulk of studies in A549 cells were performed using 1,25-D₃, studies in H460 cells were performed using EB 1089, which tends to be more stable for prolonged periods in solution. Additionally, in terms of potential clinical utility, EB1089 has reduced calcemic effects (Prudencio J, et al. 2001).

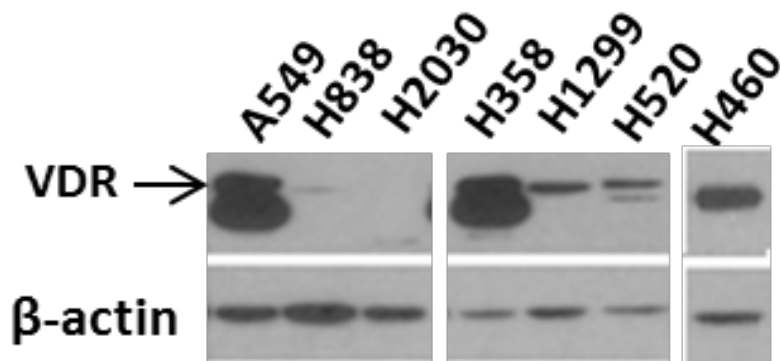


Figure 3.1. Vitamin D receptor Status in NSCLC cell lines. VDR status in selected non-small cell lung cancer cell lines by Western blotting. Top band represents VDR.

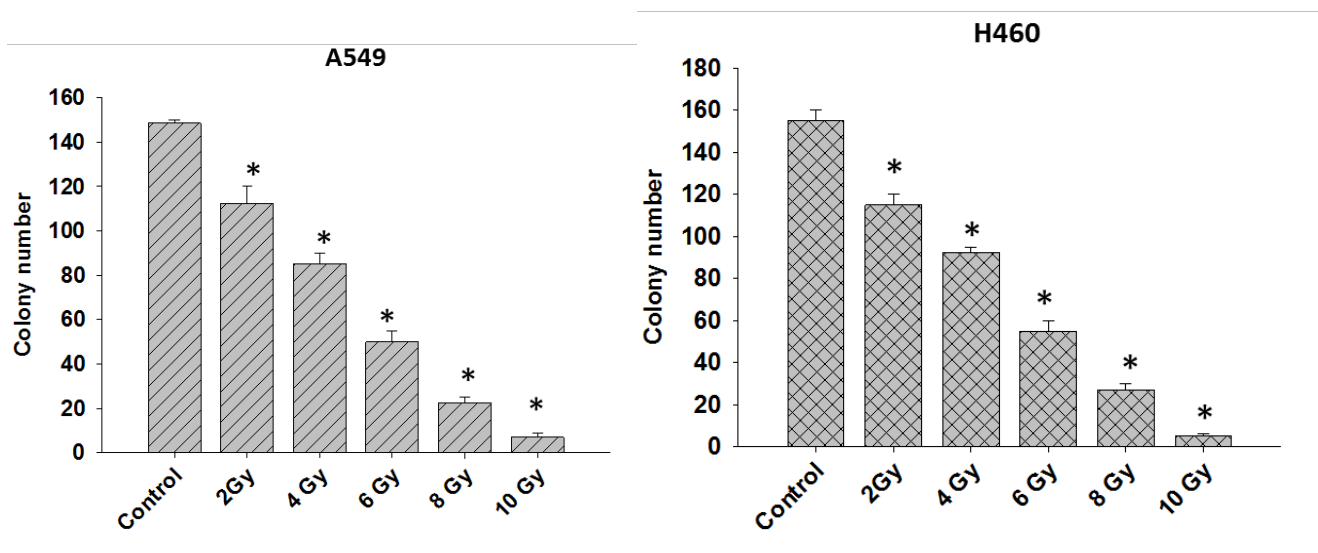


Figure 3.2. Radiation dose selection. Clonogenic survival studies were conducted with different doses of radiation in A549 and H460 cells to determine an appropriate dose to use for the studies. 200 cells/well were seeded for both A549 and H460 cell lines and studies were carried out for a span of two weeks (n=3, mean ± SE, *p<0.05 compared to control (0Gy)).

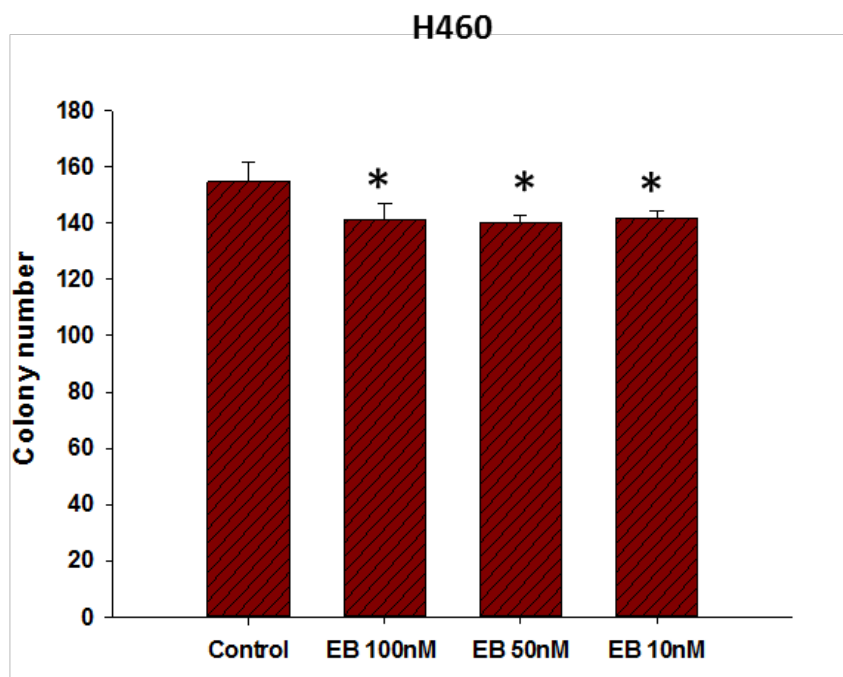
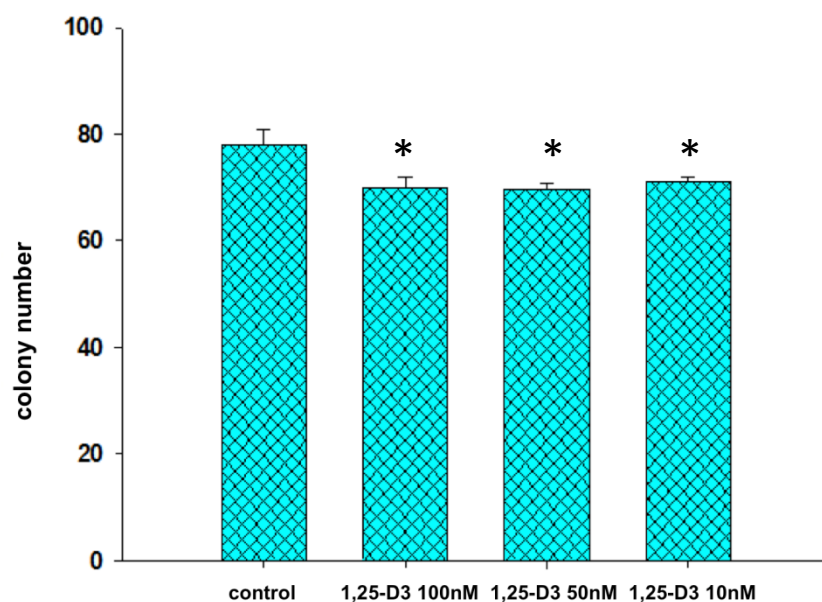


Figure 3.3. Modest effects of 1,25-D₃ or EB 1089 on cell survival. Clonogenic survival studies were conducted with different doses of 1,25-D₃ and EB 1089 in A549 and H460 cells. These studies indicate minimal inhibitory effects of 1,25-D₃ and EB 1089 by themselves on survival. For A549 cell line 150 cells/well were seeded and for H460 cell line 200 cells were seeded. Studies were carried out for two weeks (n=3, mean ± SE; *p<0.05 compared to control)

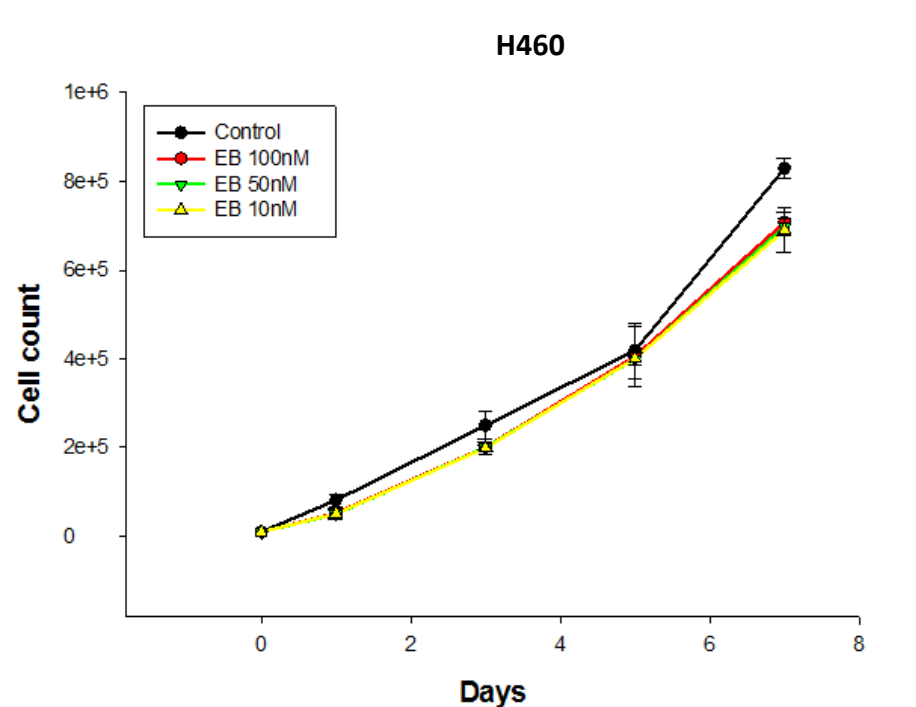
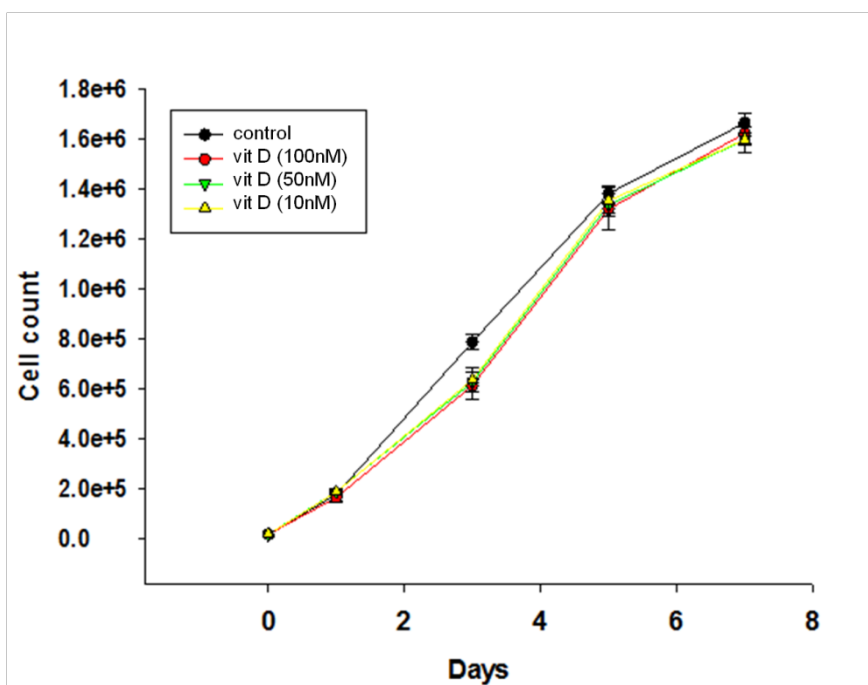


Figure 3.4. Modest effects of 1,25-D₃ or EB 1089 on cell viability. Cell viability studies were conducted with different doses of 1,25-D₃ and EB1089 in A549 and H460 cells indicates minimal inhibitory effects on survival. Trypan blue exclusion assay was employed and studies were carried out for 7 days (n=3, mean ± SE).

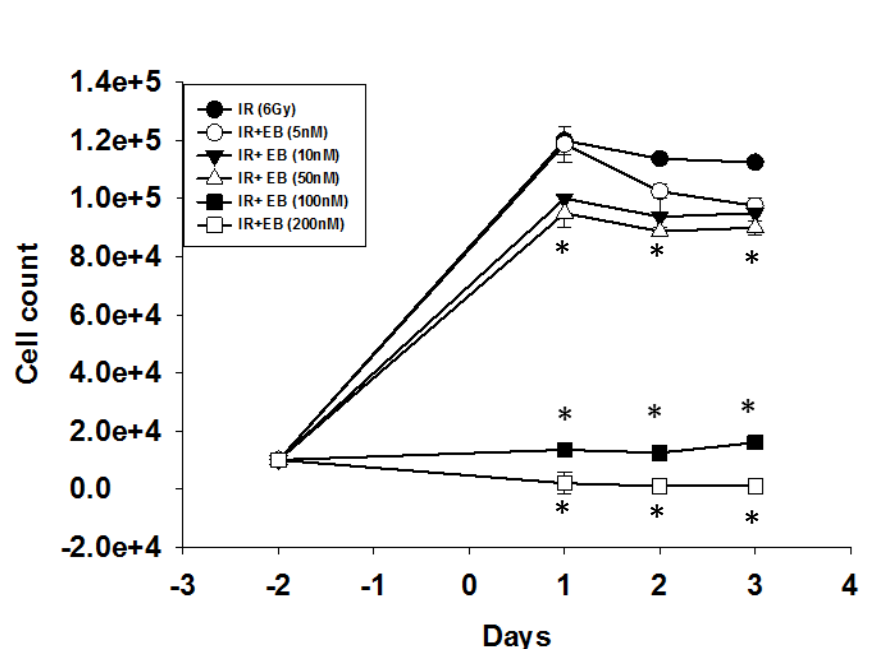
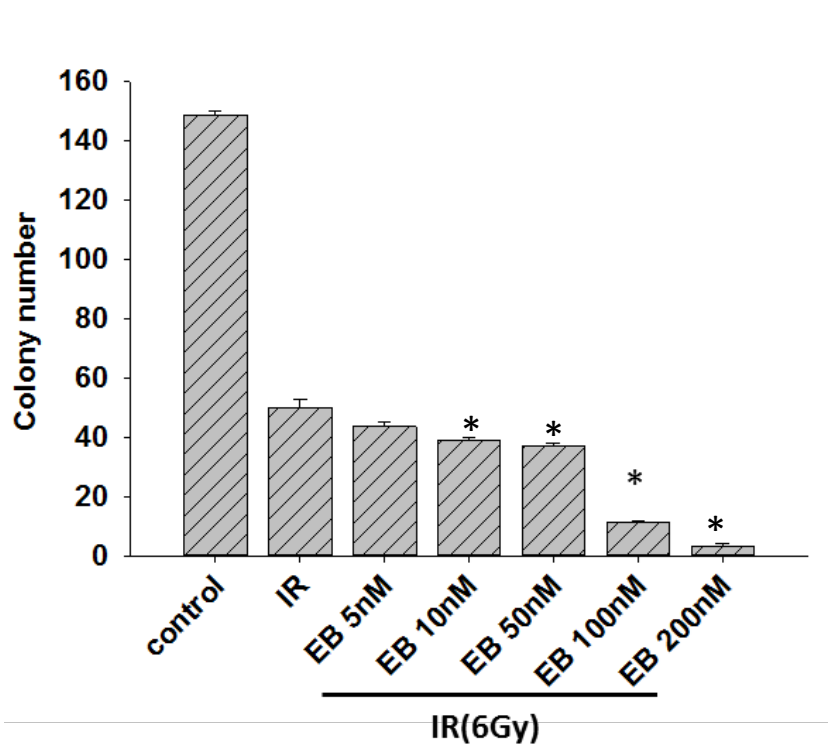


Figure 3.5. Dose response curve for the combination treatment of IR and EB 1089.

Clonogenic survival (**upper panel**) and cell viability studies (**lower panel**) were conducted with different doses of EB 1089 in combination with IR (6Gy) in H460 cells. Combination of 100 nM and 200 nM EB 1089 with 6Gy radiation dose sensitized the cells significantly (n=3, mean \pm SE, *p<0.05; compared to IR alone).

3.2 Vitamin D sensitizes lung tumor cells to radiation:

Clonogenic survival studies were performed with a single dose of radiation (6Gy) with a pretreatment of 24hrs with 100 nM of 1,25-D₃/EB 1089. Figure 3.6 indicates that in both cell lines, H460 and A549, the combination of IR and EB1089/1,25-D₃ produced a significant reduction in survival/reproductive capability of cells compared to radiation treatment alone.

To assess the effects of the combination treatment of radiation + 1,25-D₃/EB 1089 on cell viability, studies were performed using trypan blue exclusion in the A549 and H460 NSCLC cell lines. 1,25-D₃ and EB 1089 alone did not have any growth inhibitory effects on the cells as shown previously in Figure 3.4 upper and lower panels. Figure 3.7 indicates that radiation (IR) alone produced significant inhibition of cell growth; however, after the initial growth arrest between days 3 to 6, cells started to show recovery at later time points starting at day 7 and going up to day 9 in both A549 and H460 cells. The combination treatment of IR and 1,25-D₃/EB 1089 produced a more pronounced inhibition of cell growth and perhaps more importantly, dampened the proliferative recovery at later time points in both A549 and H460 cell lines as shown in lower panels of figure 3.7.

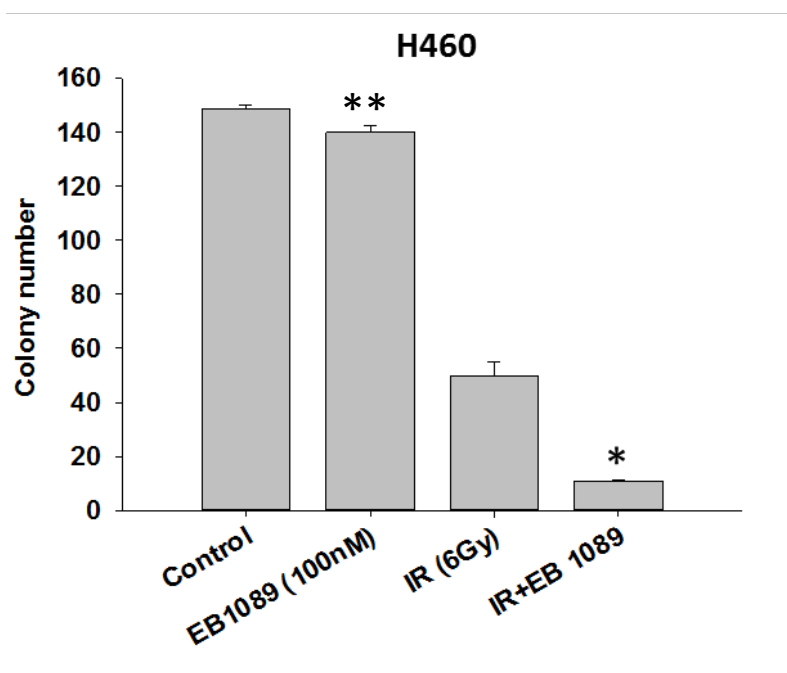
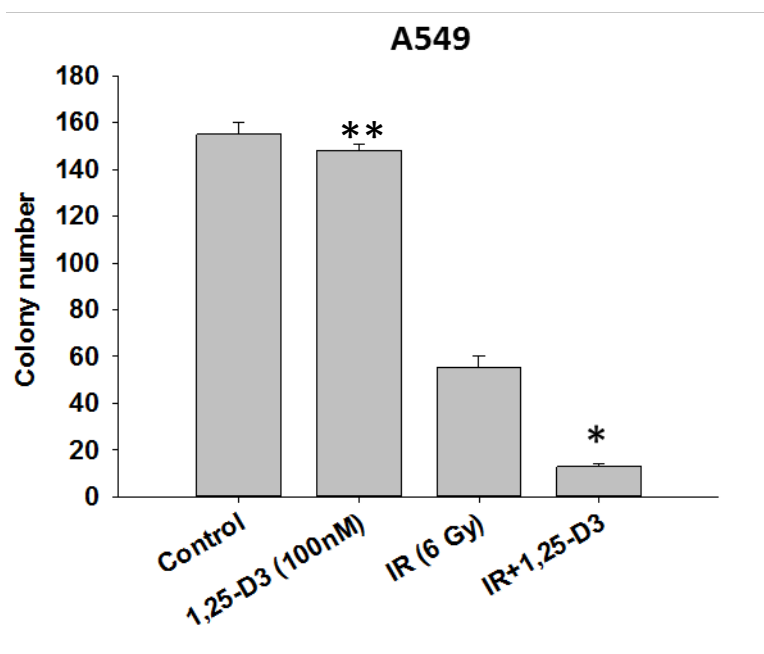


Figure 3.6. Radiosensitization of NSCLC cells by 1,25-D₃/EB 1089. Clonogenic survival studies were conducted with a single dose of 6Gy radiation (IR) alone or in combination with 100 nM 1,25-D₃ or 100 nM EB 1089 in A549 and H460 cells (n=3, mean \pm SE, *p<0.05, values are compared to IR treatment; **p<0.05, values compared to control). These studies were carried out for a time period of 2 weeks.

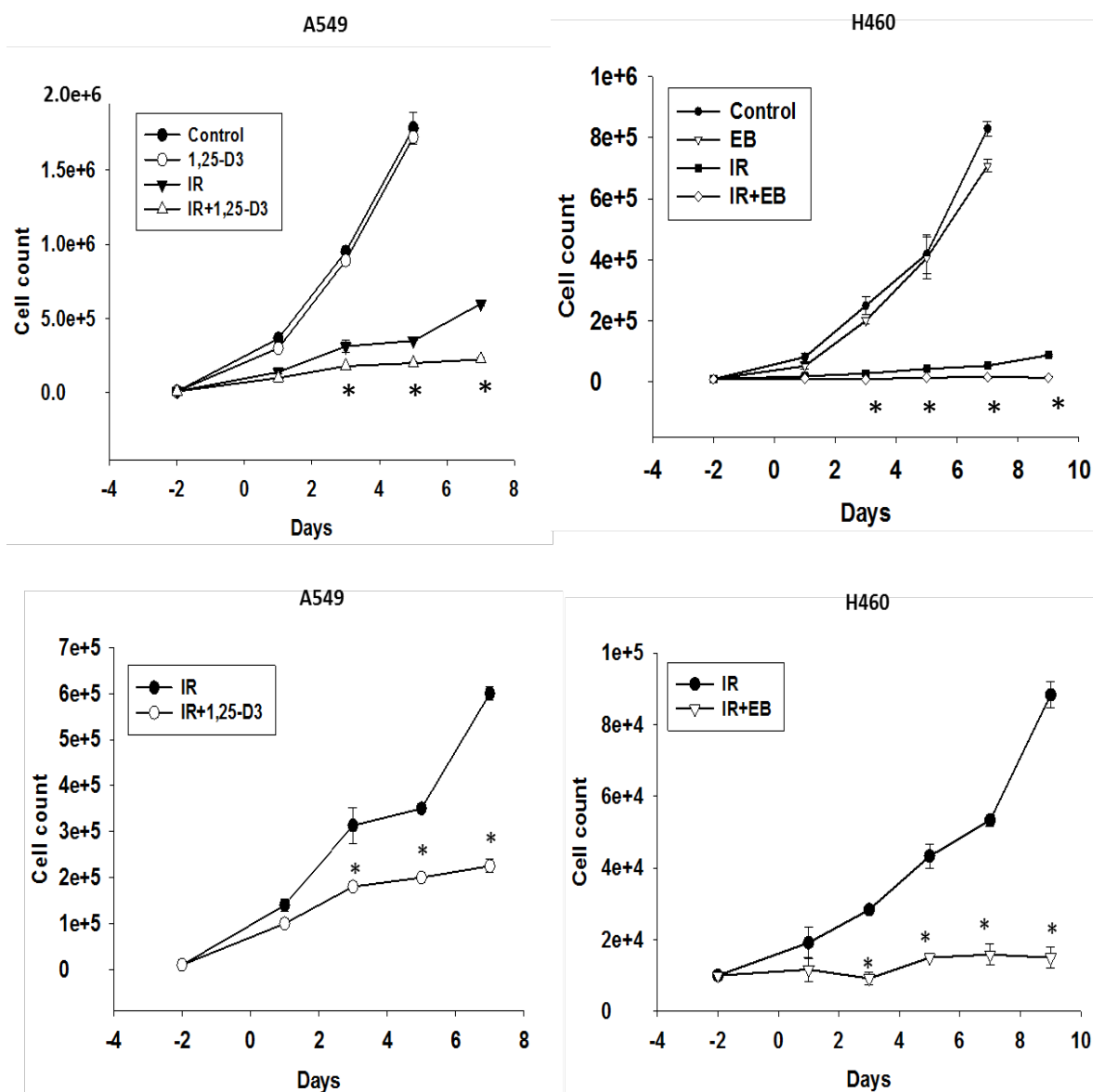


Figure 3.7. 1,25-D3/EB 1089 increases radiosensitivity and inhibits proliferative recovery of NSCLC Cells. Cell viability studies were conducted with a single dose of 6Gy radiation (IR) alone and in combination with 100 nM 1,25-D₃ or 100 nM EB 1089 in A549 and H460 cells (n=3, mean \pm SE, *p<0.05 compared to IR). Lower panel are expanded versions of the figures shown in the upper panel. Cells were radio-sensitized and significant amounts of growth inhibition was observed with the combination treatment day 3 onwards.

3.3 Sensitization limited to cells with wt/functional p53 and VDR.

Vitamin D sensitization studies were extended to other non-small cell lung cancer cell lines, specifically H358 cells, which have mutant p53 and H838 cells that are wild type in p53 but, which do not express vitamin D receptor. Unlike the outcome of studies in H460 and A549 cells, which have wild type/functional p53, 1,25-D₃ failed to sensitize H358 (mutant p53) cells to radiation as shown in figure 3.8 (upper panel). IR treatment alone inhibited growth of both cell lines, as would be expected. However, the combination treatment of IR and 1,25-D₃ did not produce a growth inhibitory effect greater than that produced by IR alone.

Similar studies were also conducted in the H838 non-small cell lung cancer cell line, which lacks a functional vitamin D receptor. Since vitamin D requires VDR to be able to influence cell function we expected that 1,25-D₃ would fail to sensitize the H838 cells. Figure 3.8 (lower panel) indicates that the combination treatment of IR+1,25-D₃ was no more effective than IR treatment alone.

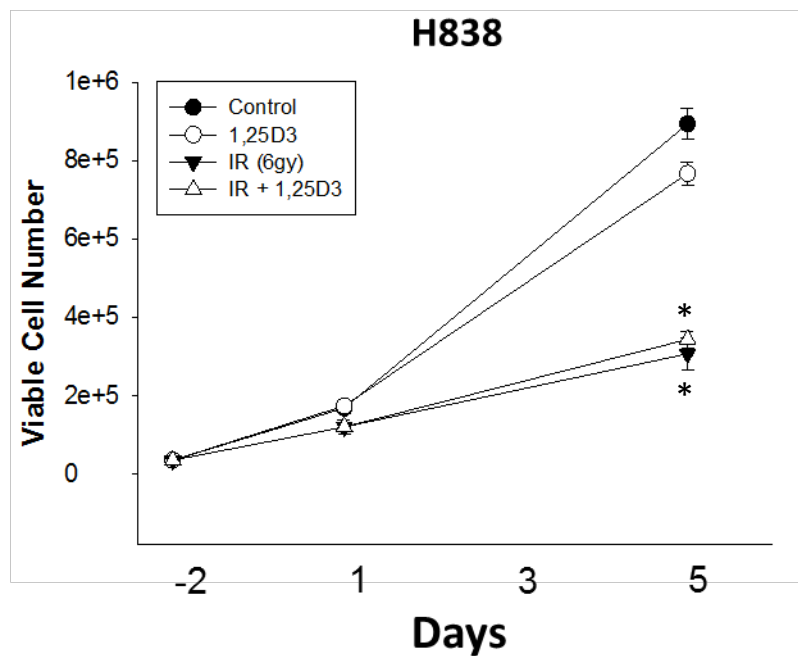
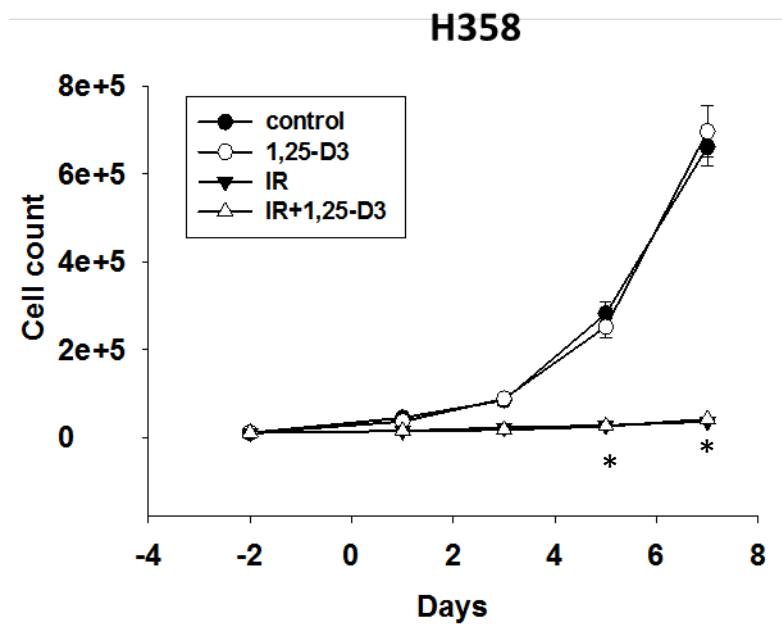


Figure 3.8. Lack of sensitization in p53 mutant and VDR lacking NSCLC cells. Cell viability studies conducted in H358 (upper panel) cells that express the VDR but are mutant in p53. Cell viability studies conducted in H838 cells (lower panel), which lack the VDR but are wild-type in p53. (n=3, mean \pm SE; *p<0.05 compared to control). The combination treatment did not sensitize the cells to radiation indicating the requirement of both wt p53 and VDR in order to attain radio-sensitization.

3.4 Summary.

1,25-D₃ or EB 1089 alone did not have any inhibitory effects on the growth (or survival) of the non-small cell lung tumor cells, A549 and H460. However, 1,25-D₃ and EB 1089 enhanced the sensitivity of these cells to radiation when used prior to radiation treatment. We have previously observed similar effects with vitamin D in breast tumor cell lines, MCF7 and ZR75 (Bristol ML, et al. 2012; Wilson EN, et al. 2011). However, cells that are mutant in p53 were not sensitized by the combination treatment. These findings are consistent with previous studies where MDA-MB-231 breast tumor cells, which lack functional p53, were not sensitized to radiation by 1,25-D₃ or the vitamin D analogue EB 1089 (DeMasters GA, et al. 2006; Bristol ML, et al. 2012). Furthermore, we confirmed the requirement for the VDR in order for sensitization to occur. Taken together, these studies indicate a requirement for both wtp53 and the vitamin D receptor for sensitization to radiation by 1,25-D₃/EB 1089.

Chapter 4

Identifying the basis of radio-sensitization.

4.1 1,25-D₃ and the vitamin D analogue EB1089 fail to increase the extent of DNA damage or interfere with DNA repair in non-small cell lung cancer cells:

In our previous studies with breast tumor cells, we failed to find evidence for increased DNA damage or decreased DNA repair associated with sensitization to radiation by 1,25 D₃/EB 1089 (Sundaram S, et al. 1999; Gewirtz DA, et al. 2002; Demasters GA, et al. 2006). It is quite common for cells to undergo DNA damage upon irradiation. We assessed DNA damage in H460 cells by performing flow cytometry to measure levels of the phosphorylated DNA repair marker γ H2AX in response to radiation and radiation + EB 1089. γ H2AX is a sensitive marker to measure double strand DNA breaks and phosphorylation (at ser139) by upstream sensors, specifically ATM for ionizing

radiation and ATR for UV radiation. Breaks were monitored at 1 hour, when DNA damage is expected to be maximal and at 48 hours, when repair is likely to be complete. As shown in Figure 4.1, high levels of damage were observed at the earlier time points, but declined by the 8 hour time point in both A549 and H460 cells. Furthermore, neither the initial extent of damage nor the rate of decline (i.e. repair) was affected by the presence of 1,25-D₃ or EB 1089.

4.2 Minimal induction of apoptosis or necrosis by radiation and radiation + 1,25 D₃/EB 1089 in NSCLC cells.

To further obtain insight into how the sensitization might be occurring, studies were conducted to assess apoptosis and necrosis in response to treatment with IR alone and IR + EB 1089 by flow cytometry using Annexin V/PI staining in H460 and A549 cells starting from Day 1 up to Day 7. Staurosporine, a protein kinase inhibitor, which is known to induce apoptosis, was used as a positive control (Bertrand R, et al. 1994; Watson SP, et al. 1988). AnnexinV/PI staining can distinguish cells undergoing early apoptosis, late apoptosis and necrosis. Phosphatidyl serine is found on outer membrane of the cells when they are undergoing early apoptosis. Annexin V molecules, which are bound to a fluorescent FITC, have affinity for phosphatidyl serine, and bind to these molecules and then fluoresce. Early apoptotic cells stain positive only for annexin V. However, when cells are in their late apoptotic state, the cell membrane begins to break open, which causes PI (an intercalating agent) to enter the cell. Late apoptotic cells stain positive for both annexin V and PI. In addition, cells that become necrotic stain positive only for PI since annexin V along with other cellular components is degraded during necrosis. As shown in Figures 4.2 and 4.4, there was minimal

induction of apoptosis or necrosis by radiation alone and this was not increased to any significant extent by the combination treatment of radiation and 1,25-D₃/EB 1089 in A549 and H460 cells. Quantification of results is represented in upper panels of figures 4.3 and 4.5.

Although annexinV/PI staining is an accurate and relatively specific assay for apoptosis, we sought to confirm (the relative absence of) apoptosis by assessing poly (ADP-ribose) polymerase (PARP) cleavage using western blotting. PARP is activated during DNA damage and is cleaved by active caspases. Again, Staurosporine was used as a positive control. As indicated in the lower panels of figures 4.3 and 4.5, the lack of PARP cleavage for both radiation and the combination treatment of IR and EB 1089 confirmed our previous finding in both A549 and H460 cells.

.

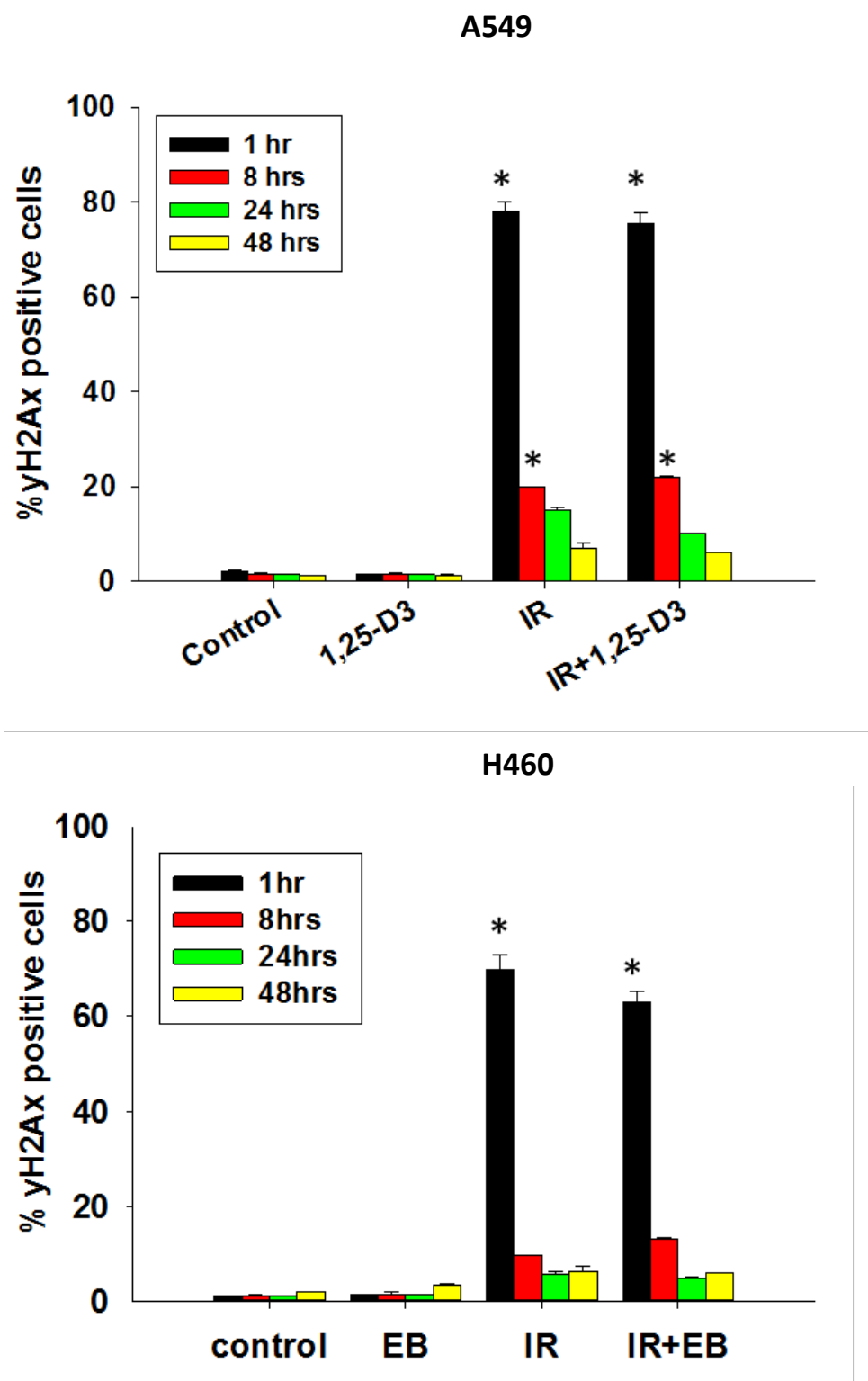


Figure 4.1. Induction and repair of DNA damage by IR and 1,25-D₃/EB 1089 + IR. Induction and decline of DNA damage in A549 (upper panel) and H460 (lower panel) cells by FACS analysis of γH2AX levels after treatment with 100 nM 1,25-D₃ and EB 1089 and 6Gy radiation alone and in combination (n=3 mean ± SE; *p<0.05, values are compared to control). Phosphorylation of γH₂AX was observed to be induced as early as 1 hour and went down soon after by 8 hours.

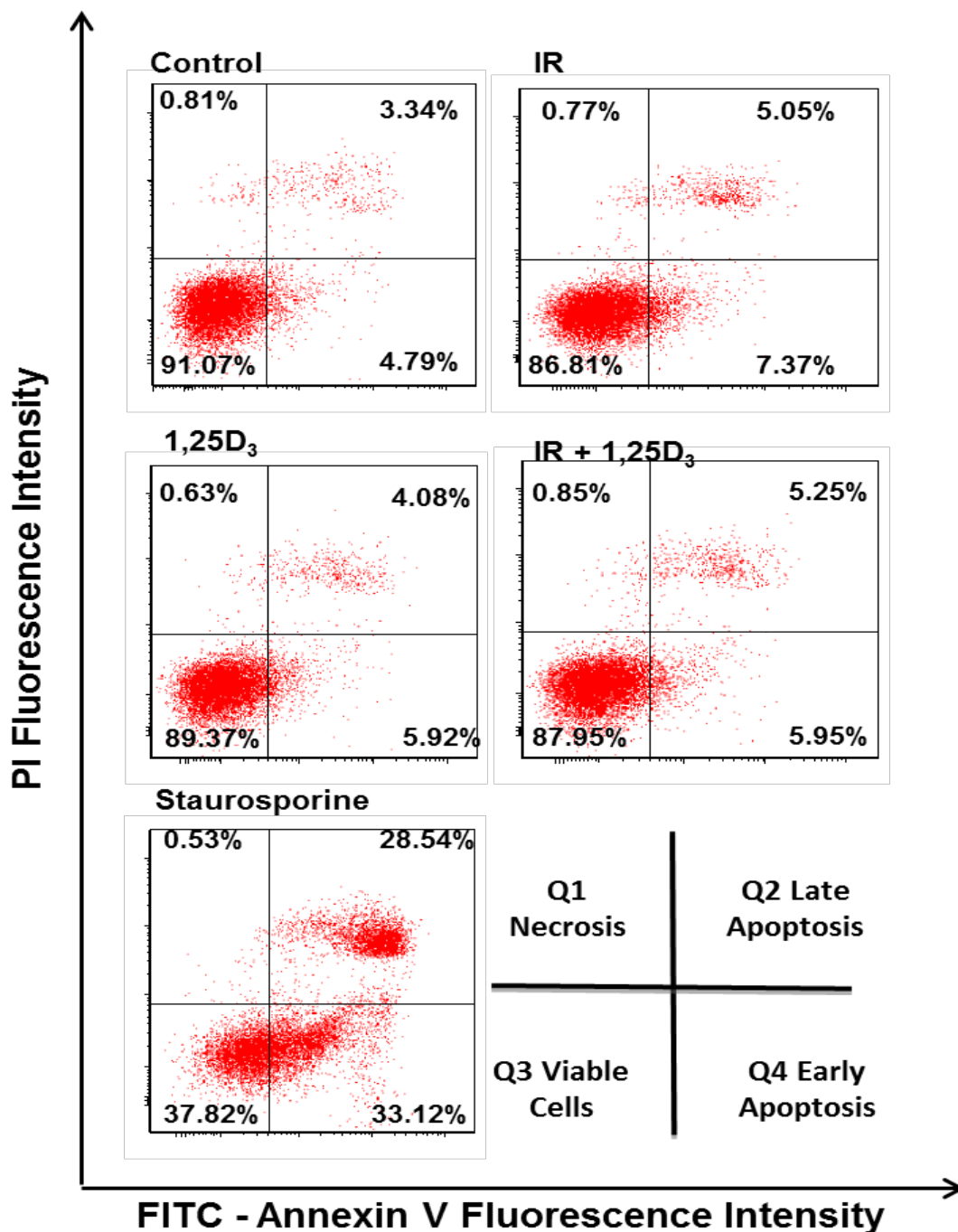


Figure 4.2. Assessment of apoptosis (and necrosis) induction in A549 cells by flow cytometry. Quantification of apoptosis in A549 cells by flow cytometry using annexinV/PI staining. Quadrants indicating both early and late apoptosis were taken into account. Staurosporine, which induces apoptosis by activating caspase3, was used as a positive control. These histograms show day 3 of a representative experiment.

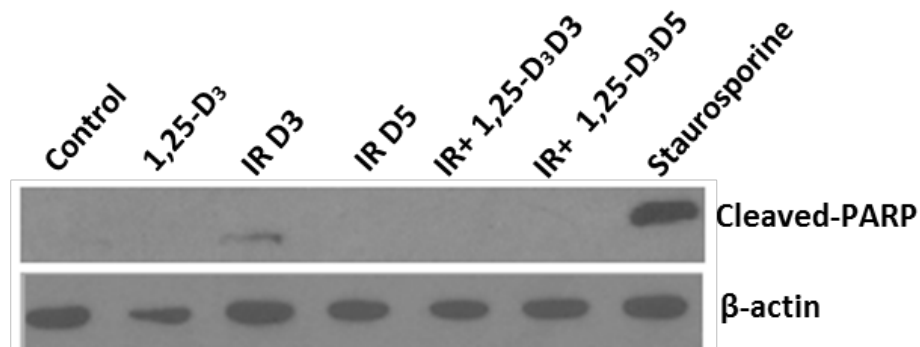
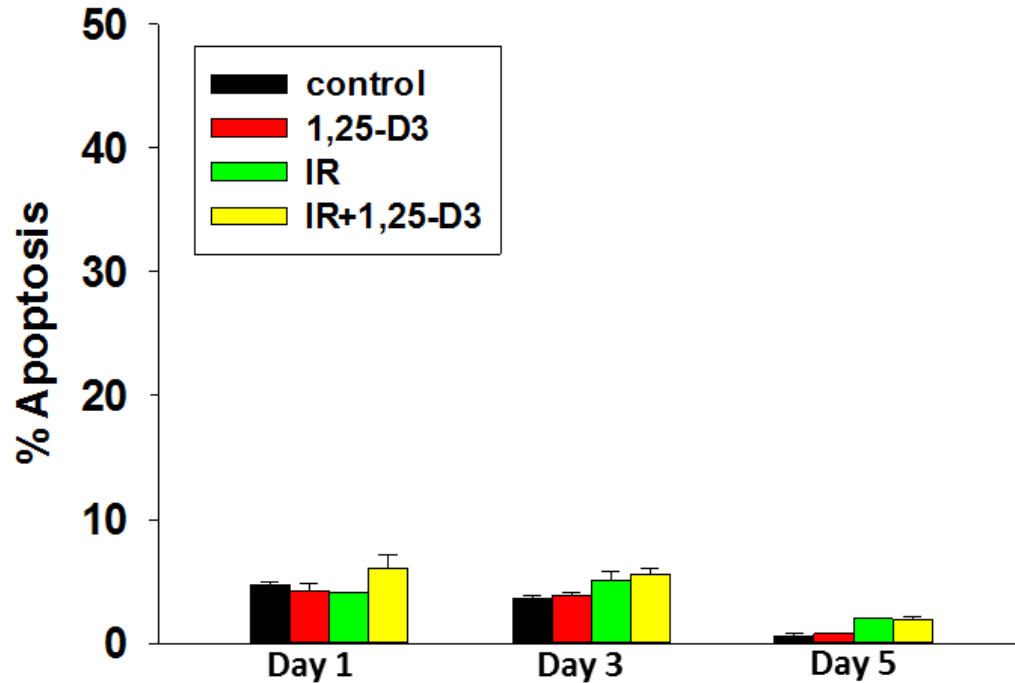


Figure 4.3. Minimal increase in apoptosis in response to radiation sensitization in A549 cells. Representation of apoptosis quantification in A549 cells(n=3, mean \pm SE). No significant increase in apoptosis was observed with any of the treatments (upper panel). PARP cleavage assessed by Western blotting on Days 3 and 5 (lower panel). Staurosporine was used as a positive control. Except for a slight band for IR Day3, no PARP cleavage was observed at other time points with different conditions.

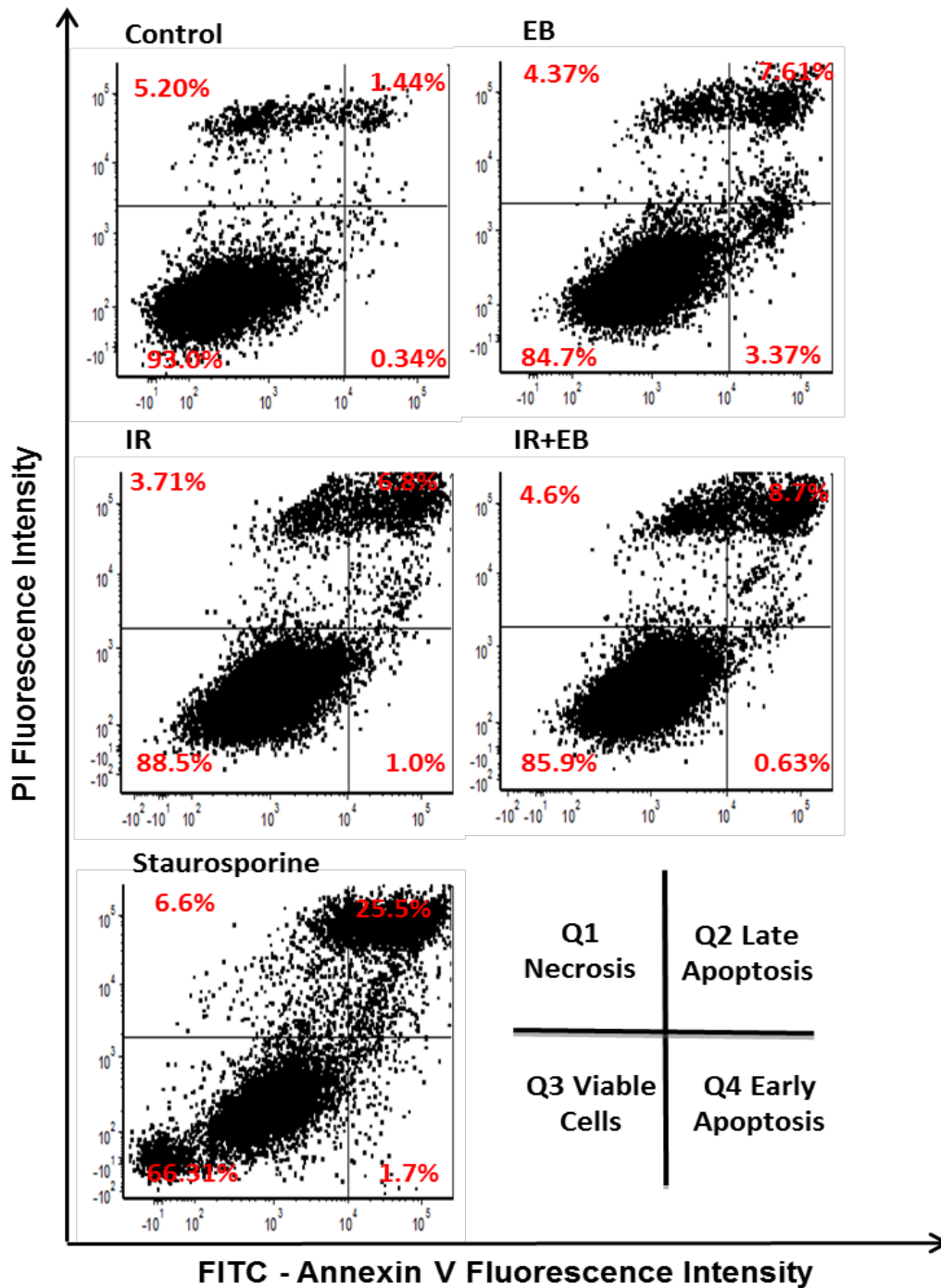


Figure 4.4. Assessment of apoptosis (and necrosis) induction in H460 cells by flow cytometry. Quantification of apoptosis in H460 cells by flow cytometry using annexin V/PI staining. Quadrants indicating both early and late apoptosis were taken into account. Staurosporine, which induces apoptosis by activating caspase3, was used as a positive control. These histograms show day 3 of a representative experiment.

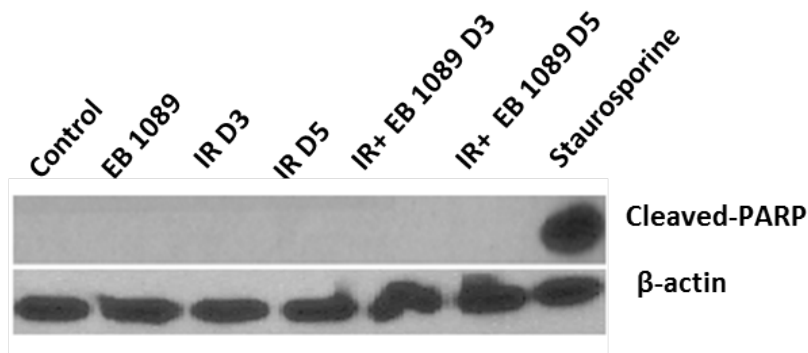
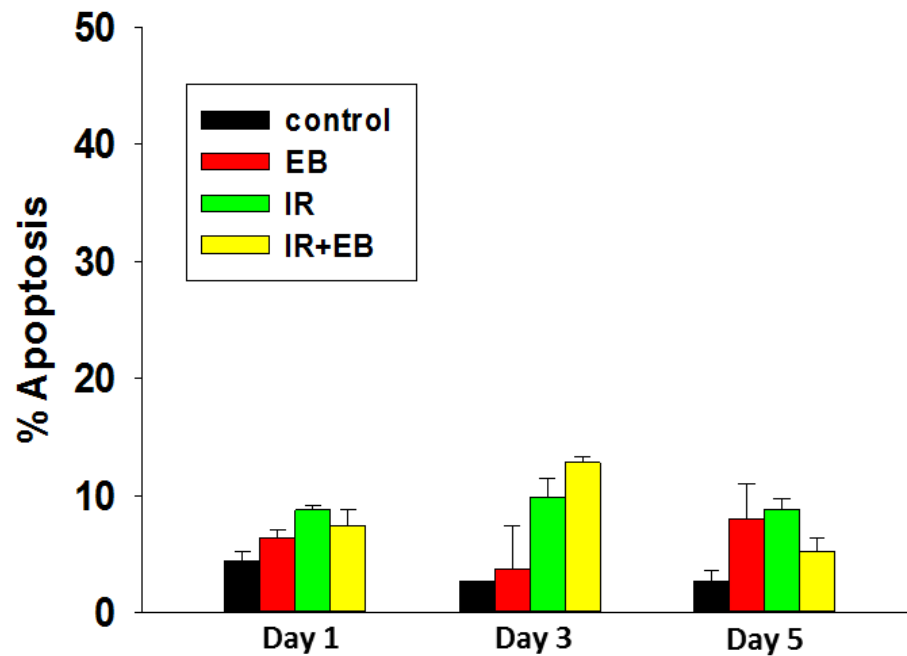


Figure 4.5. Minimal increase in apoptosis in response to radiation sensitization in H460 cells. Representation of apoptosis quantification in H460 cells($n=3$, mean \pm SE). No significant increase in apoptosis levels was observed for various treatment conditions (upper panel). PARP cleavage assessed by Western blotting (lower panel) on Days 3 and 5. Staurosporine was used as a positive control. No significant PARP cleavage was observed for any of the treatment conditions.

4.3 1,25-D₃ /EB 1089 fail to increase the extent of senescence induction by radiation in NSCLC cells.

Since the primary response observed either with radiation alone or radiation + 1,25 D₃/EB 1089 appeared to be growth arrest, and since we previously demonstrated that the primary response to radiation in p53 wild type cells is senescence (Jones KR, et al. 2005; Gewirtz DA, et al. 2008) in the absence of significant apoptosis, we evaluated whether the extent of senescence might have been altered for the combination treatments.

To assess levels of senescence in A549 and H460 cells, we employed β -galactosidase staining (Dimri GP, et al. 1995) and x-gal, a chromogenic substrate, which when cleaved by β -galactosidase leads to formation of an insoluble blue color compound. The extent of blue color positive cells indicates senescence. The β -galactosidase staining images shown in figures 4.6 and 4.8 for A549 and H460 cells indicate that both radiation treatment alone and the combination of radiation with 1,25-D₃ or EB 1089 lead to an increase in senescence levels compared to control and 1,25-D₃/EB 1089 treated cells. Alterations in morphology are another indication of senescence. Cells undergoing senescence become enlarged and flattened and form neuron-like arm projections from cells as indicated by β -galactosidase staining images for both A549 and H460 cells.

Flow cytometry was employed to quantitatively measure the extent of senescence in our cell models utilizing a fluorogenic substrate C₁₂FDG to stain the senescent positive cells (Debacq-Chainiaux F, et al. 2009). C₁₂FDG is a membrane impermeable non fluorescent molecule which becomes trapped and produces green fluorescence when

hydrolyzed by β -galactosidase. Figures 4.7 and 4.9 respectively provide quantification of the time dependent increase in senescence induced by radiation and the combination treatments in A549 (figure 4.7) and H460 (figure 4.9) cell lines. These studies indicate an increase in senescence levels but fail to demonstrate any significant differences between radiation treatment alone and the combination of radiation + 1,25 D₃/ EB 1089 in terms of the extent of senescence.

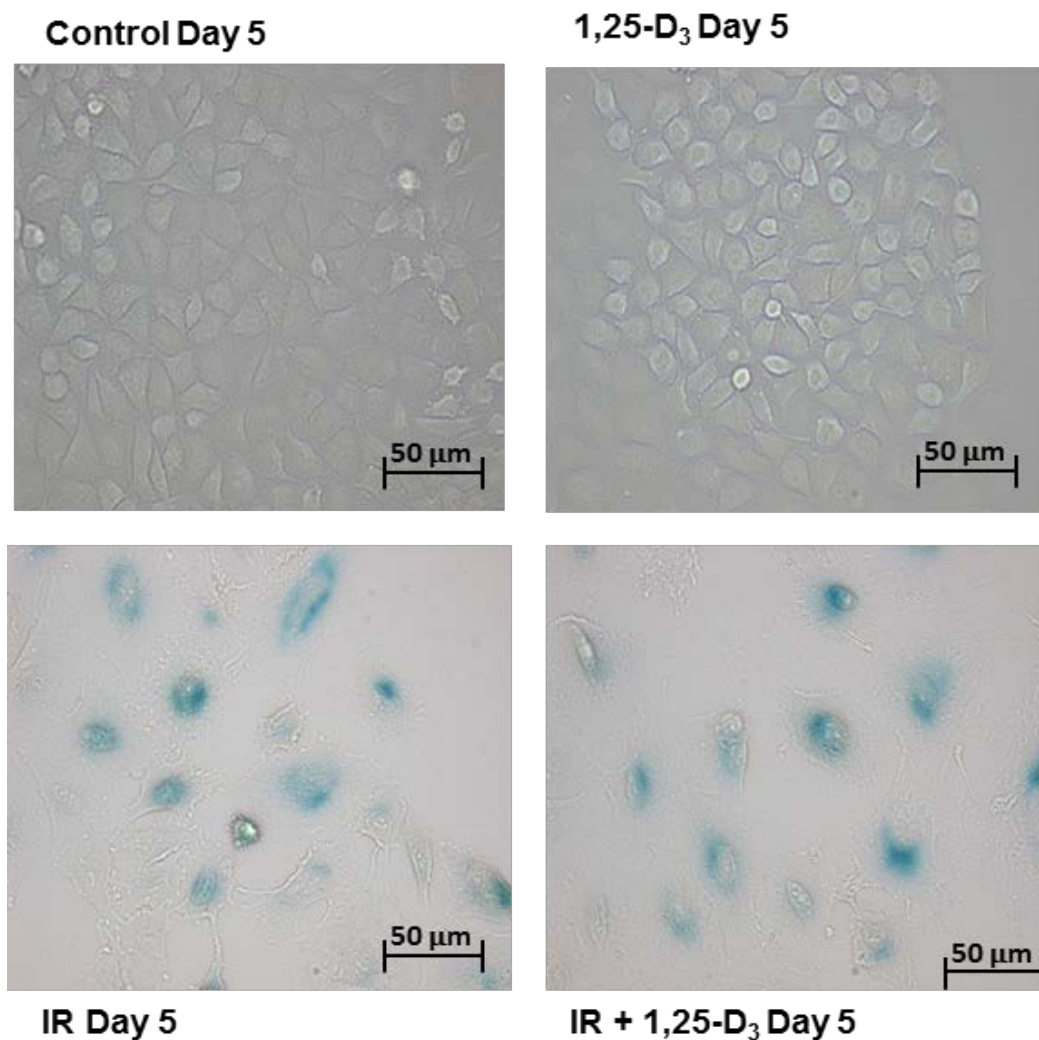


Figure 4.6. Assessment of senescence induction by radiation and radiation + 1,25D₃ in A549 cells. β -galactosidase staining indicative of senescence in A549 cells after 6Gy radiation alone and the combination of IR (6Gy) + 1,25-D₃. All images were taken at the same magnification. Extensive induction of senescence is observed in response to IR and IR+1,25-D₃ treatments as indicated by an increase in insoluble blue color compound formed by cleavage of x-gal by β -galactosidase. Images shown are from a representative experiment.

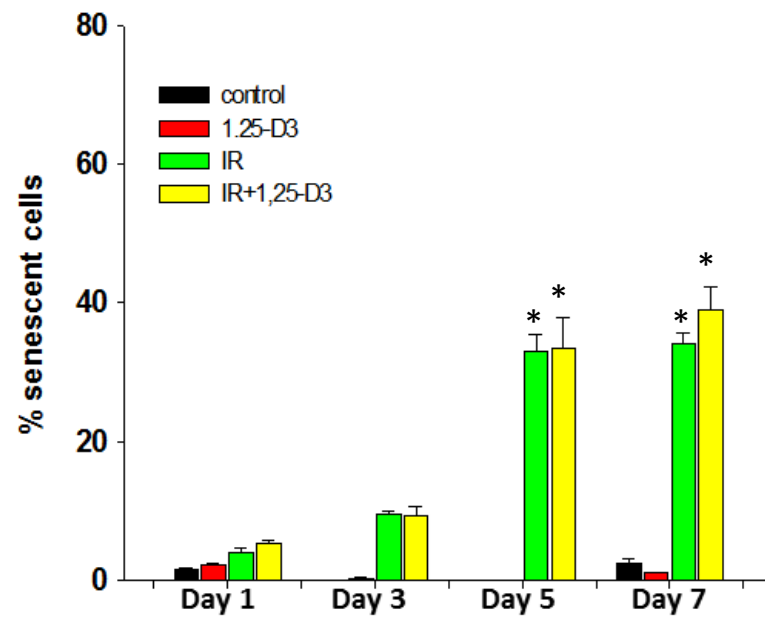
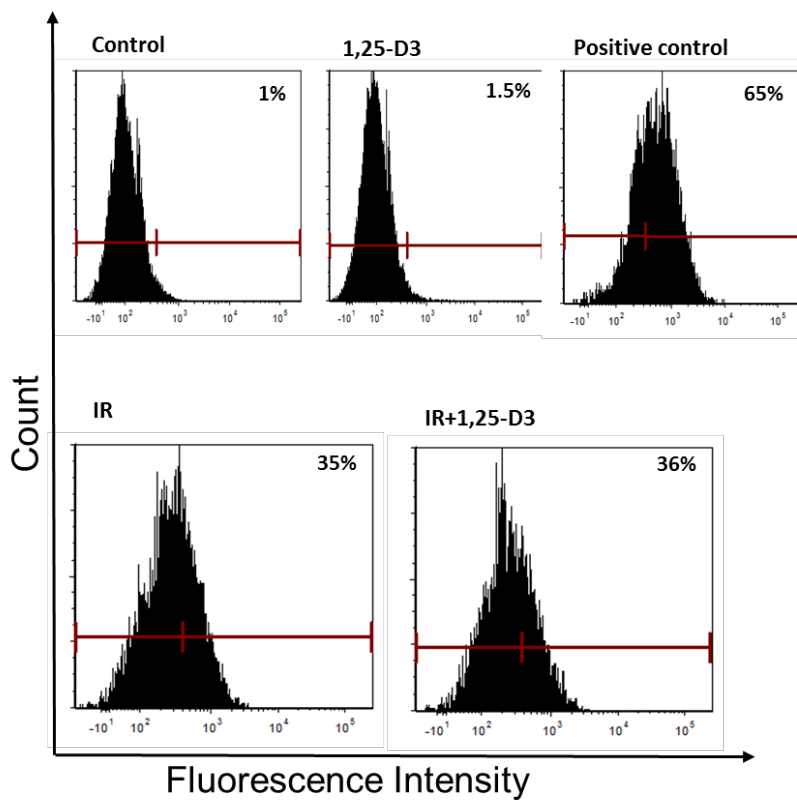


Figure 4.7. No significant increase in senescence by IR+1,25-D₃ compared to IR in A549 cells. Quantification of senescence by flow cytometry using fluorogenic substrate C₁₂FDG in A549 cells. (n=3, mean \pm SE; *p<0.05 compared to control). Fluorescent intensity produced by hydrolysis of this substrate by β -galactosidase enzyme was measured by flow cytometry. Adriamycin used as a positive control. Histograms in the upper panel show day 3 of a representative experiment.

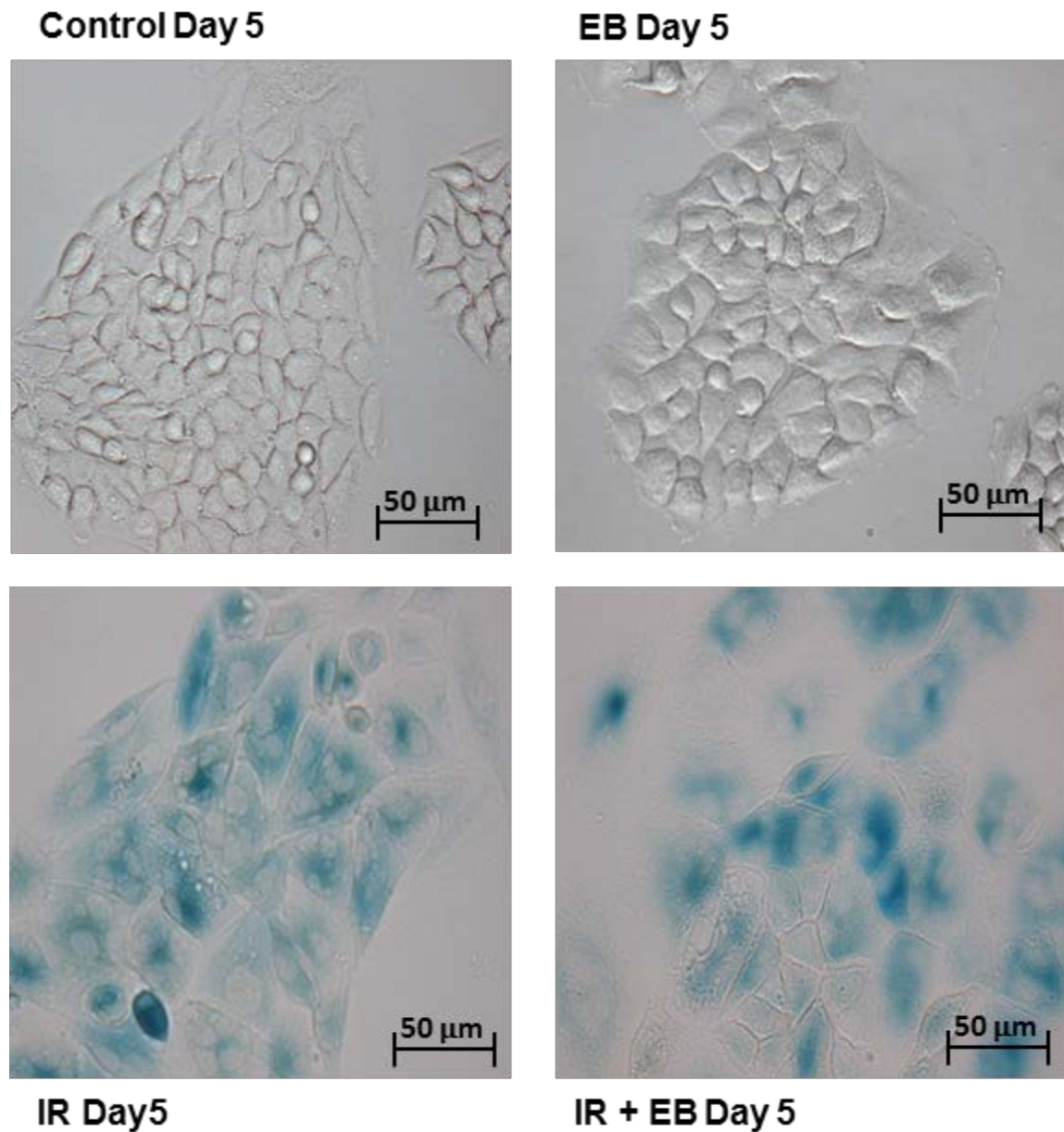


Figure 4.8. Assessment of senescence induction by radiation and radiation + EB 1089 in H460 cells. β -galactosidase staining indicative of senescence in H460 cells after 6Gy radiation alone and the combination of IR (6Gy) + EB 1089. All images were taken at the same magnification. Extensive induction of senescence is observed in response to IR and IR+1,25-D₃ treatments as indicated by an increase in insoluble blue color compound formed by cleavage of x-gal by β -galactosidase. Images shown are from a representative experiment.

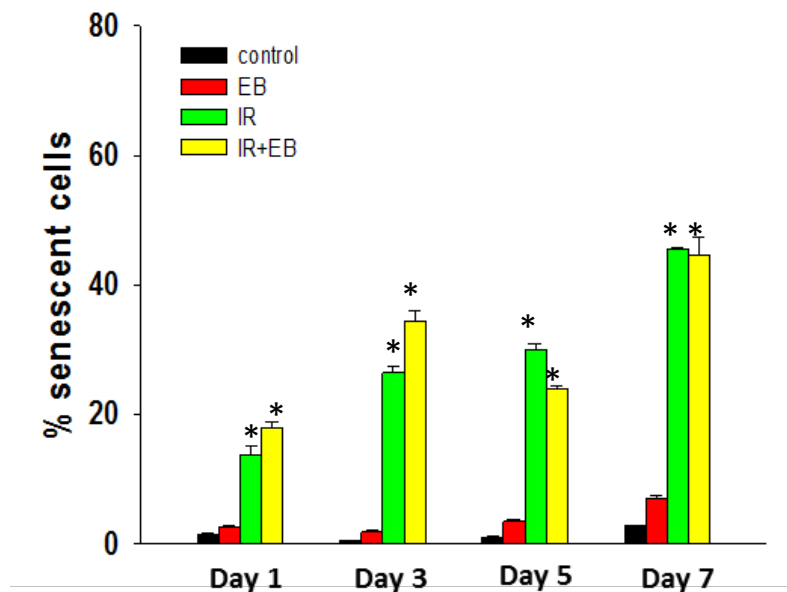
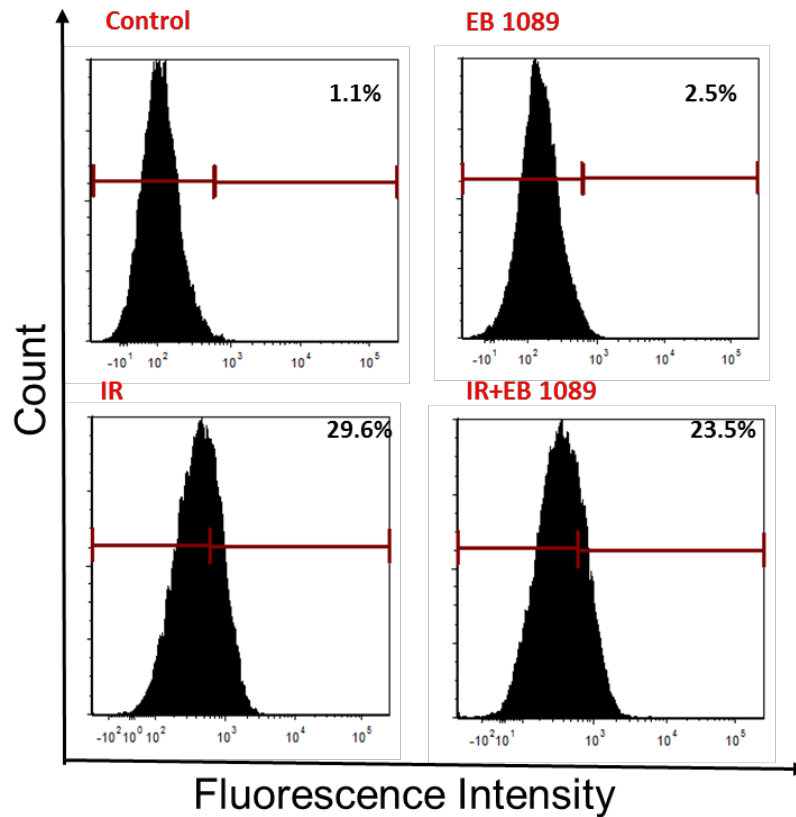


Figure 4.9. No significant increase in senescence by IR+EB 1089 compared to IR in H460 cells. Quantification of senescence by flow cytometry using a fluorogenic substrate, C₁₂FDG in H460 cells. (n=3, mean \pm SE). Fluorescent intensity produced by hydrolysis of this substrate by β -galactosidase enzyme was measured by flow cytometry. Adriamycin used as a positive control. . Histograms in the upper panel show day 3 of a representative experiment.

4.4 Evidence that autophagy is the mode of radio-sensitization by 1,25-D₃/EB 1089.

We have previously reported quite extensively on the capacity of 1,25 -D₃ and vitamin D analogs to sensitize breast tumor cells to radiation (Sundaram S, et al. 1999; Gewirtz DA, et al. 2002; Sundaram S, et al. 2003; Bristol ML, et al. 2012; Wilson EN, et al. 2011; Demasters GA, et al. 2006). In recent work, we have confirmed that this sensitization, a decline in viable cell number, occurs through the promotion of autophagy based on reversal of sensitization through either pharmacological or genetic interference with autophagy (Bristol ML, et al. 2012; Wilson EN, et al. 2011).

In the current studies to investigate whether autophagy is the mode of sensitization in A549 and H460 cells, the cells were stained with acridine orange and acidic vesicle formation was assessed visually using fluorescence microscopy as well as quantitatively using flow cytometry. Acridine orange is a lysotropic dye, which accumulates in acidic vesicles in a pH dependent manner. It gives out green fluorescence at neutral pH but becomes protonated and trapped in acidic vesicles and gives out bright orange fluorescence. Figures 4.10 show acridine orange images captured for A549 and H460 at day 3. Increased acidic vesicle formation is observed with the combination treatment of IR and 1,25-D₃ in A549 cells. To quantify the amount of autophagy in the system, flow cytometry was used to measure the fluorescent intensity of acridine orange. Figures 4.11 show time dependent increase in acidic vesicle formation for radiation treatment alone and the combination of radiation and 1,25-D₃ in A549 cell line. We observed significant differences between IR and IR+1,25-D₃ induced autophagy in A549 cells. Serum starvation was used as a positive control. Cells were starved for 4

hours before they were analyzed. Both imaging and flow cytometry data confirmed that there is a significant increase in autophagy levels for IR treatment and combination treatment of IR and 1,25-D₃ in A549 cells. However, as shown in Figure 4.12 and 4.13, the extent of autophagy was not significantly different for radiation alone and EB 1089 + radiation in the H460 cells.

To confirm autophagy induction, we used confocal microscopy to assess LC3 puncta staining in the cytoplasm (Kirisako T, et al. 2000). A549 and H460 cells were stably transfected with a plasmid containing LC3 labeled with green fluorescent protein (GFP). Microtubule associated protein light chain 3 (LC3) is a mammalian homolog of yeast Atg8 found in the cytoplasm in LC3I form. Upon autophagy induction, LC3 conjugates with phosphatidylethanolamine (Atg8-PE) to form LC3II, which is then targeted to autophagosome membranes (Kuma A, et al. 2007; Klionsky DJ, et al. 2012). Therefore, GFP-LC3 is observed by fluorescence microscopy either as a diffuse cytoplasmic pool in cells not undergoing autophagy or as puncta structures which essentially indicate autophagosome formation when autophagy is induced (Mizushima N, et al. 2010- methods in mammalian autophagy research). The upper panels of figures 4.12 and 4.15 show an increase in puncta formation for both radiation and the combination treatment of radiation and 1,25-D₃/EB 1089 in A549 and H460 cells. As with the acridine orange staining, we did not detect any major differences between the puncta induced by radiation alone and with the combination of radiation and EB 1089 in H460 cells.

Once it was determined that autophagy was being initiated, it was necessary to assess autophagic flux to establish if autophagy was going to completion since formation of

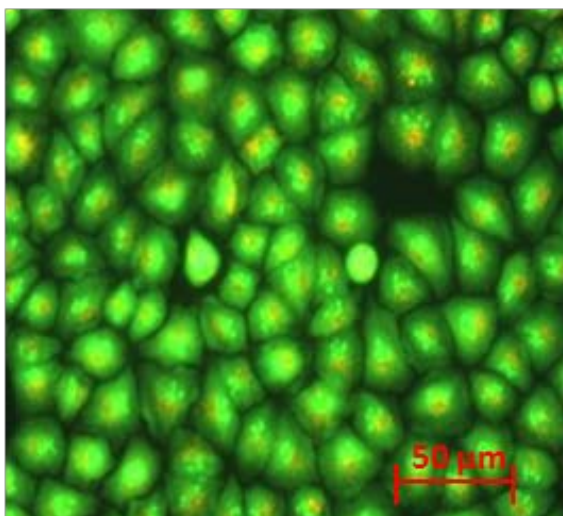
autophagic vesicle does not necessarily mean that autophagosomes are fusing with lysosomes and that the cellular components are being degraded. We employed western blotting to assess degradation of the established autophagic marker p62 (Klionsky DJ, et al. 2012; Johansen T, et al. 2009). The p62 protein serves as a link between light chain 3 (LC3) protein and ubiquitinated substrates. p62 and p62 bound ubiquitinated proteins are incorporated into autophagosomes and are degraded when autophagosomes fuse with lysosomes. Autophagic flux correlates with decreased p62 levels (Klionsky DJ, et al. 2012). Another protein marker for autophagic flux that was assessed by Western blotting is microtubule associated light chain II, which is recruited to the autophagosomal membrane during formation of the autophagosome. LC3II is also degraded by lysosomal enzymes when autophagosomes fuses with lysosomes. Both p62 and LC3II appear to be degraded over time for both radiation treatment and the combination treatment of radiation and 1,25-D3/EB 1089 compared to no treatment and EB1089 treatment alone as shown in figures 4.12 and 4.15 (lower panels). These data further indicate that there was no apparent difference in the extent of autophagic flux between radiation alone and the combination of radiation and 1,25-D3/EB 1089, indicating that autophagic flux is unlikely to be a distinguishing characteristic. LC3II bands appear to degrade slower than p62 because LC3II is present both outside and inside the autophagosomes due to which even when LC3II inside the autophagosome starts to degrade, there is some present on the outside of the membrane which degrades later in the end.

These studies suggest that although we see a significant increase in autophagy levels with the combination treatment of radiation and 1,25-D₃ as compared to IR alone in

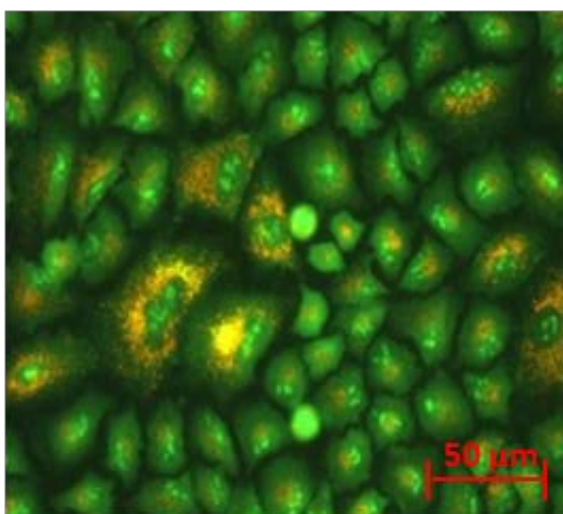
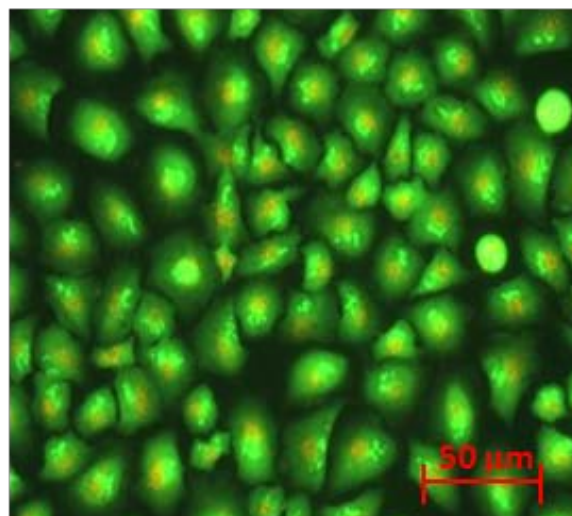
A549 cells, there is only a modest difference in autophagy levels for radiation alone and the combination of IR and EB1089 in H460 cells arguing *against the simple explanation that sensitization might be caused solely by an increase in the extent of autophagy.*

There is evidence in literature indicating that autophagy can play more than one functional role in response to different treatment conditions (Dutta D, et al. 2013; Zhang TZ, 2014; Gewirtz DA, et al. 2014) Studies show that it can either be cytoprotective or cytotoxic in nature. This prompted us to determine what functions was autophagy playing in response to IR and IR+EB 1089 in the NSCLC cell model.

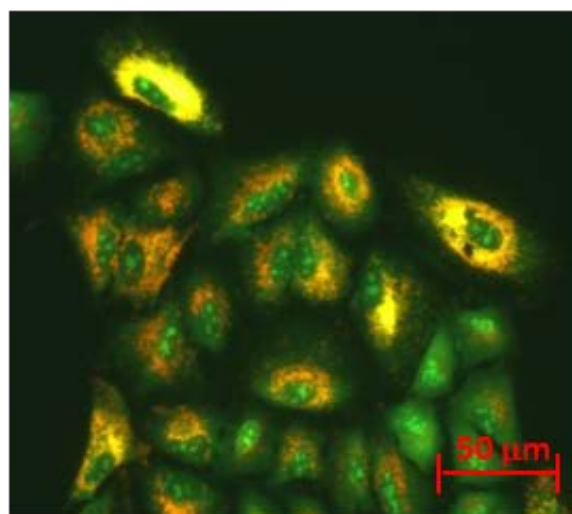
Control Day 3



1,25-D₃ Day 3



IR Day 3



IR + 1,25-D₃ Day 3

Figure 4.10. Analysis of autophagy induction by acridine orange staining in A549 NSCLC Cells. Acridine orange staining indicate induction of autophagy in A549 cells by radiation (IR, 6 Gy) alone or radiation+1,25-D₃. All images were taken at the same magnification. Extensive amounts of Autophagy induction is observed for both IR and the combination treatment of IR+1,25-D₃ as indicated by high amounts of orange/red colored autophagic vesicles.

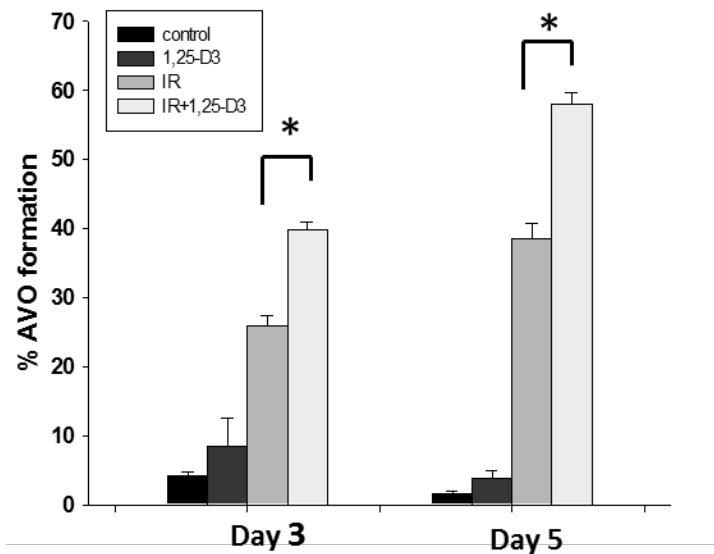
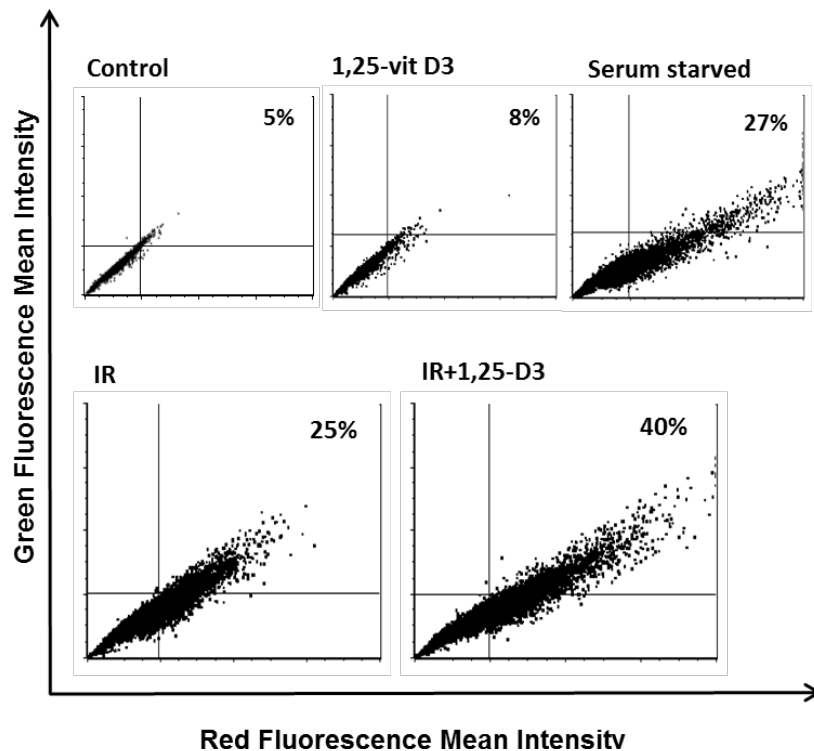


Figure 4.11. Assessment of autophagy by flow cytometry in A549 NSCLC Cells. Quantification of autophagy by Flow cytometry in A549 cells using acridine orange staining. (n=3, mean \pm SE, *p<0.05). Serum starved cells (4 hours) were used as positive control. Time dependent increase in autophagic vacuolar organelle formation was observed in response to both treatments. However the increase in autophagy levels with the combination is significantly higher than IR treatment alone. . Histograms in the upper panel show day 3 of a representative experiment.

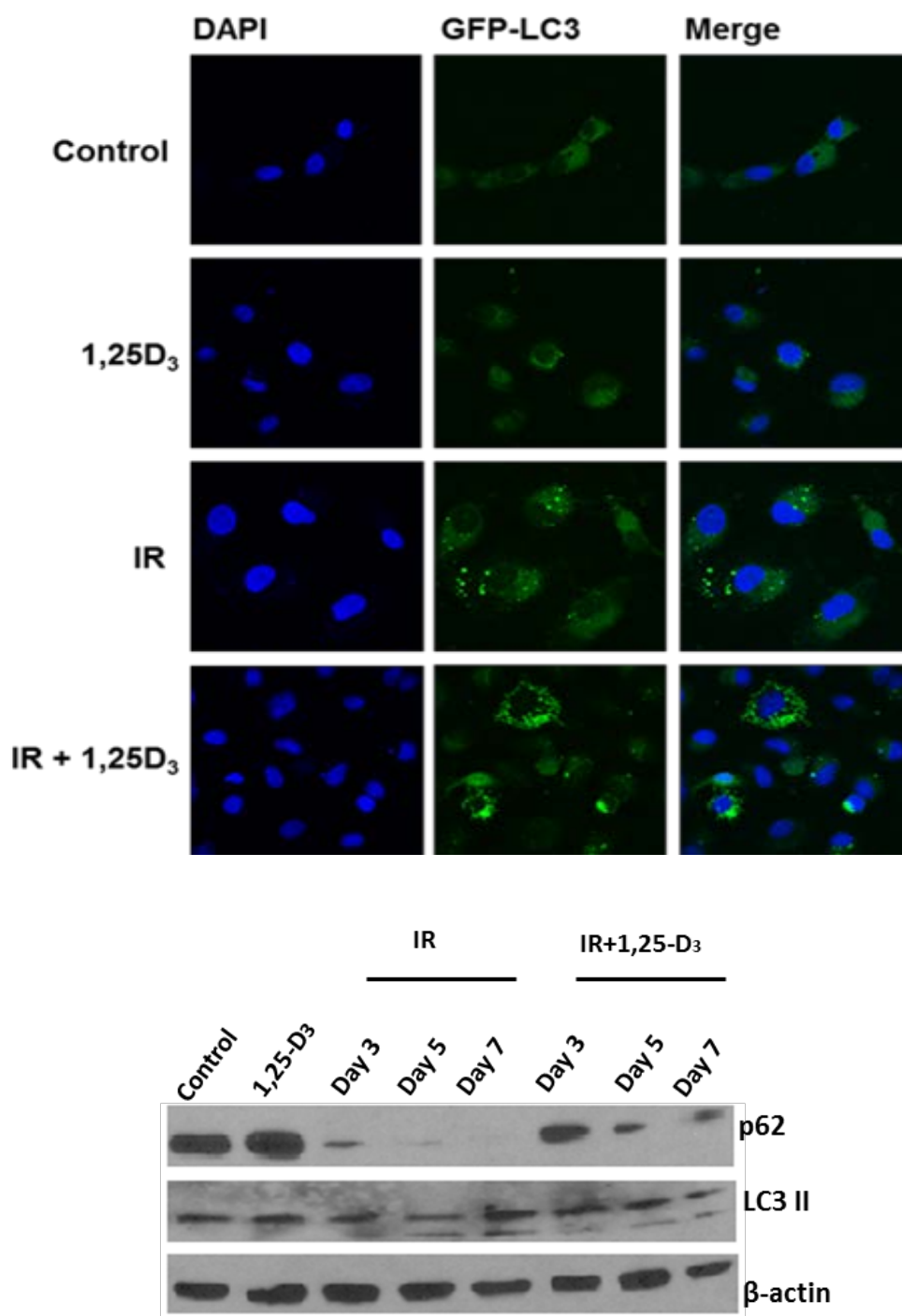


Figure 4.12. Assessment of autophagic flux in A549 NSCLC Cells. Assessment of GFP-LC3 puncta by confocal microscopy in A549 cells on day 3 are shown in the upper panel (all images were taken at same magnification). Increase in LC3II puncta formation was observed for both IR and the combination of IR+1,25-D₃ treatments. Assessment of autophagic flux markers, p62 and LC3II in response to different treatment conditions by Western blotting shown in the lower panel. Degradation of p62 indicate autophagic flux when cellular components in the autophagosome are broken down.

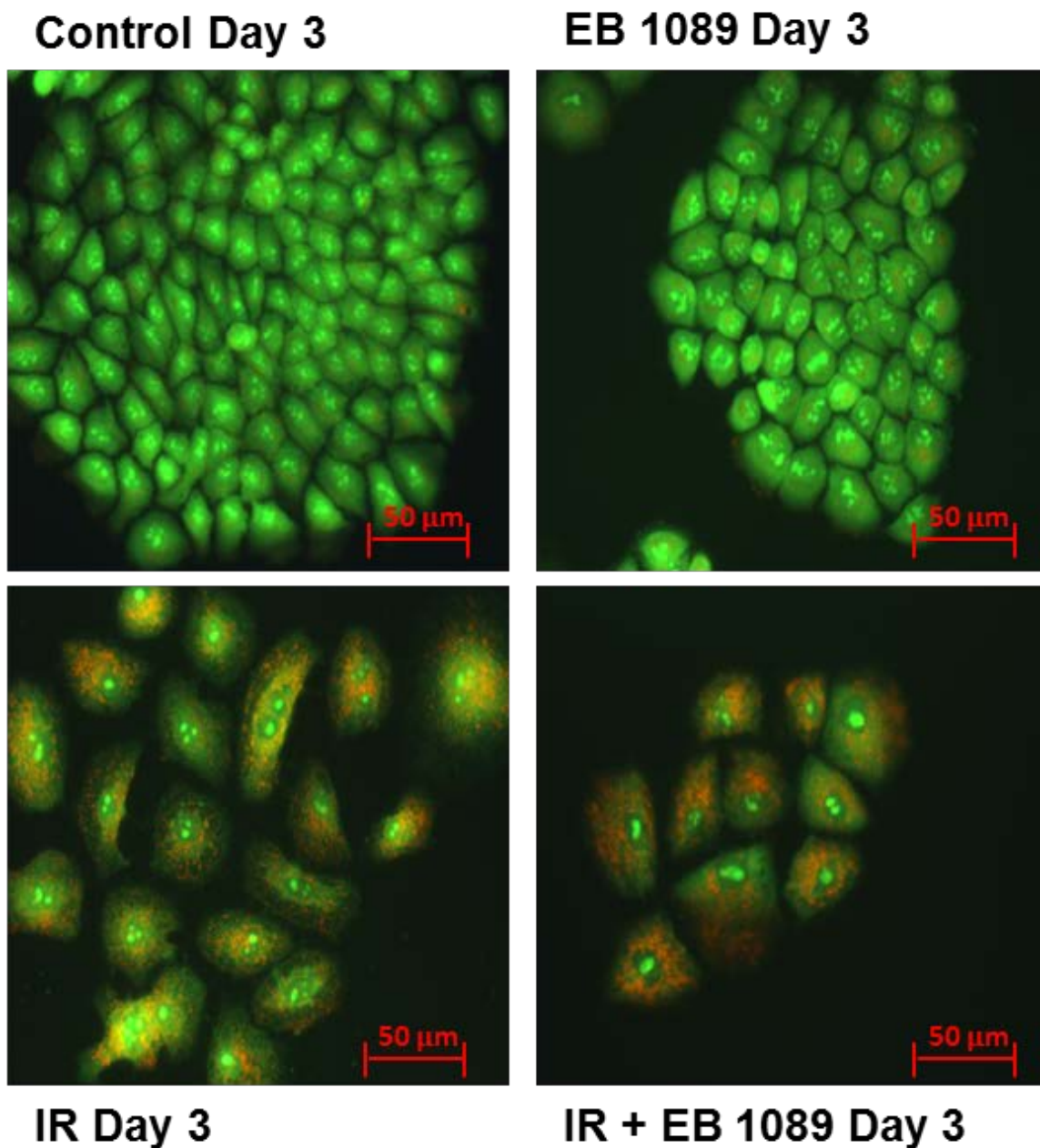


Figure 4.13. Analysis of autophagy induction by acridine orange staining in H460 NSCLC Cells. Acridine orange staining indicating induction of autophagy in H460 cells by radiation (IR, 6 Gy) alone or radiation + EB 1089. All images are taken at the same magnification. Extensive amounts of Autophagy induction is observed for both IR and the combination treatment of IR+EB 1089 as indicated by high amounts of orange/red colored autophagic vesicles.

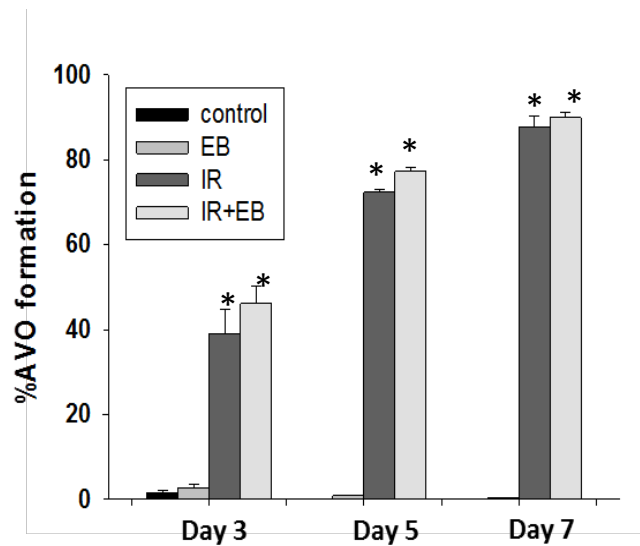
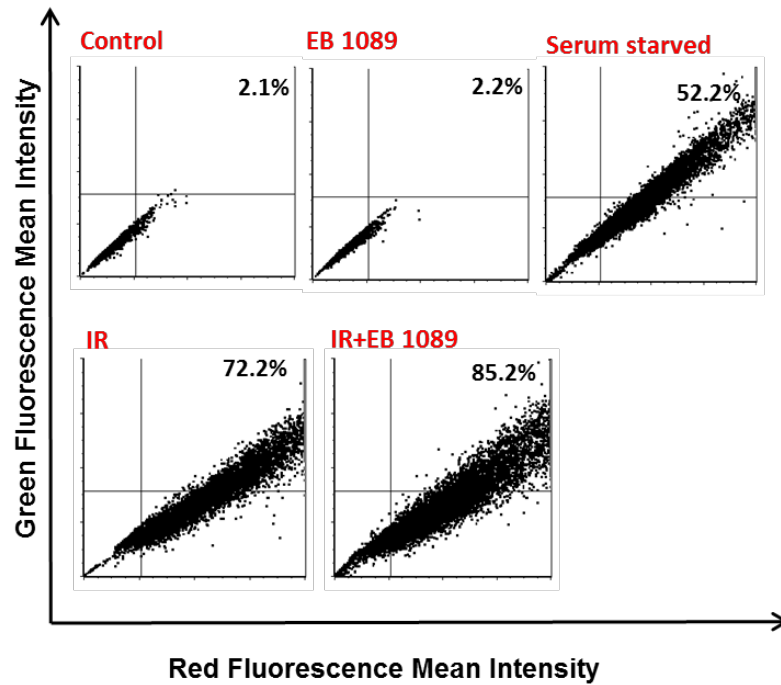


Figure 4.14. Assessment of autophagy by flow cytometry in H460 NSCLC Cells.

Quantification of autophagy by Flow cytometry in H460 cells. (n=3, mean \pm SE; *p<0.05 compared to control). Serum starved cells used as positive control. Serum starved cells (4 hours) were used as positive control. Time dependent increase in autophagic vacuolar organelles formation was observed in response to both IR and IR+EB 1089. No significant differences between IR and IR+EB 1089 were observed. . Histograms in the upper panel show day 3 of a representative experiment.

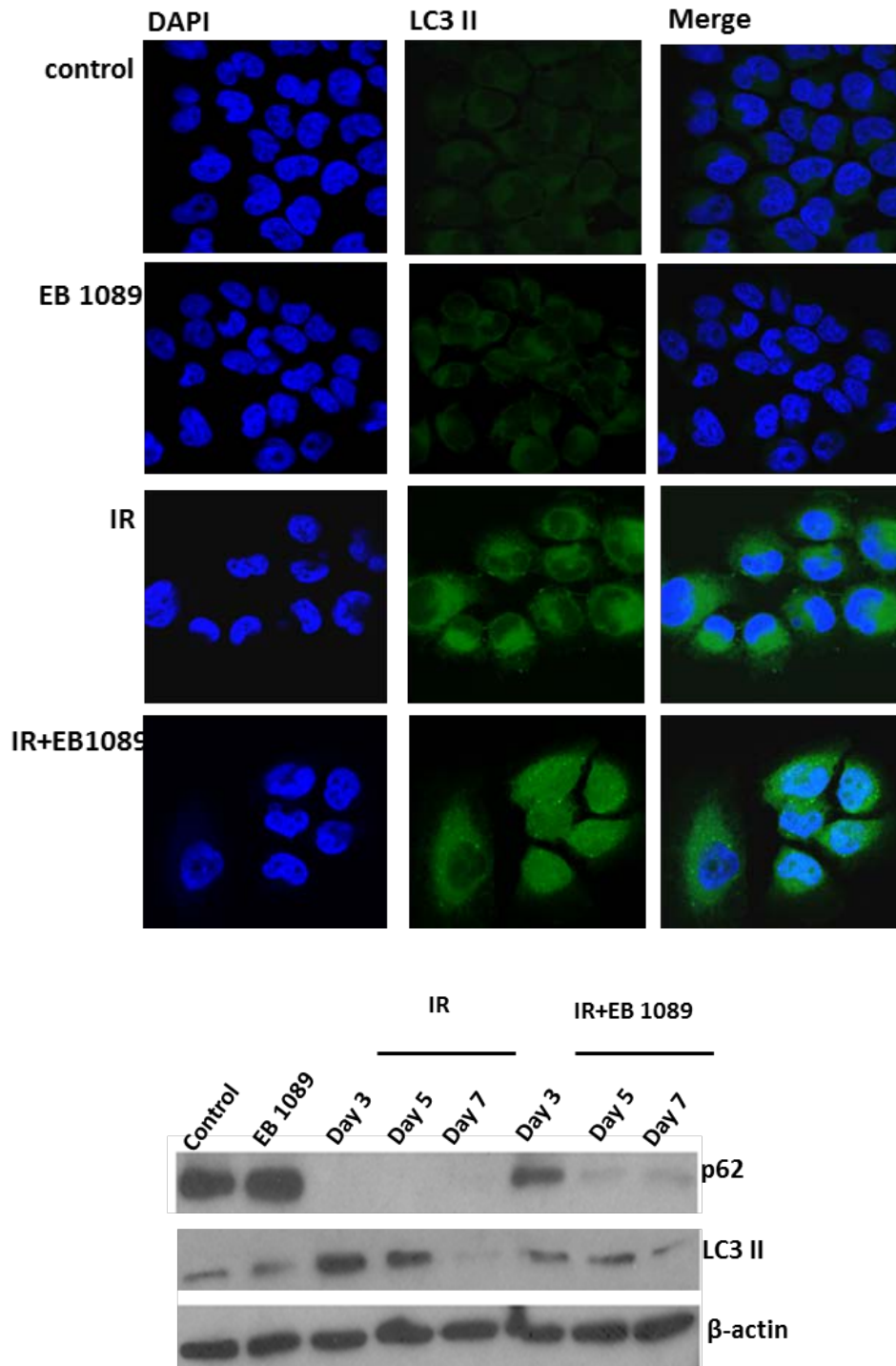


Figure 4.15. Assessment of autophagic flux in H460 NSCLC Cells. Assessment of GFP-LC3 puncta by confocal microscopy in H460 cells on day 3 are shown in the upper panel (all images were taken at same magnification). Increase in LC3II puncta formation was observed for both IR and the combination of IR+EB 1089 treatment. Assessment of autophagic flux markers, p62 and LC3II in response to different treatment conditions by Western blotting shown in the lower panel. Degradation of both p62 and LC3II indicate autophagic flux when cellular components in the autophagosome are broken down.

4.5 Autophagy induced by radiation treatment is cytoprotective in nature.

It has been established by a number of laboratories, including our own, that radiation-induced autophagy frequently has a cytoprotective function (Bristol ML, et al. 2012; Wilson EN, et al. 2011). Recent studies by Kroemer's lab have shown this to be the case in the A549 and H460 cells by demonstrating that inhibition of radiation induced autophagy increases sensitivity to radiation (Ko A, et al. 2014) and this has been confirmed in our current work. We performed cell viability studies using trypan blue after inhibiting radiation-induced autophagy using both pharmacological and genetic approaches. The pharmacological inhibitors used for this study were chloroquine and bafilomycin. Chloroquine is an anti-malarial drug which enters the acidic vacuoles by diffusion and alters the pH, which interferes with the fusion between lysosomes and autophagosomes (Klionsky DJ, et al. 2012). Bafilomycin, which is a H⁺ATPase enzyme inhibitor, works on a similar principle in that it alters the pH of the vacuoles and interferes with the activation of lysosomal enzymes (Klionsky DJ, et al. 2012; Tapper H, et al. 1997). Both chloroquine and bafilomycin are late stage autophagy inhibitors. We first performed an MTT assay using different doses of these inhibitors to determine suitable concentrations to be used for our studies, which would have minimal or (preferably) no toxic effects. Based on the results as shown in figure 4.16 and the literature we decided to use concentrations of 10 μ M for chloroquine and 100 nM for bafilomycin.

Cell viability studies were also performed to determine growth inhibitory effects of the inhibitors over a longer period of time. Figure 4.17 shows that neither of the inhibitors altered cell viability compared to untreated control cells.

Acridine orange staining was employed to confirm that autophagy was blocked using these pharmacological inhibitors by visualizing the acridine orange stained vacuoles. Autophagy inhibition was confirmed in A549 cells (figure 4.18 upper panel) and H460 (figure 4.20 upper panel) cells. Since both chloroquine and bafilomycin are late stage inhibitors of autophagy, they lead to an accumulation in acidic vesicle since they block lysosome from fusing to autophagosome by altering the pH of the acidic vesicles. Furthermore, since these inhibitors alter the pH of the vesicles, the change in color from orange to yellow provides an additional confirmation of autophagy inhibition. Finally, assessing levels of the p62 protein by Western blotting confirmed autophagy inhibition in A549 (figure 4.18, lower panel) and H460 (figure 4.20, lower panel) cells. Accumulation of p62 when chloroquine and bafilomycin were used indicated that the autophagic process was prevented from going into completion.

Cell viability studies shown in Figures 4.19 and 4.21 demonstrate that a pharmacological blockade to autophagy resulted in an increase in sensitivity to radiation and a pronounced inhibition of cell growth as indicated by decrease in cell viability. Although figures 4.22, 4.23, 4.24 and 4.25 show that blocking autophagy using pharmacological inhibitors led to a significant increase in apoptosis when assessed by flow cytometry using annexinV/PI staining in A549 cells but did not do so in H460 cells.

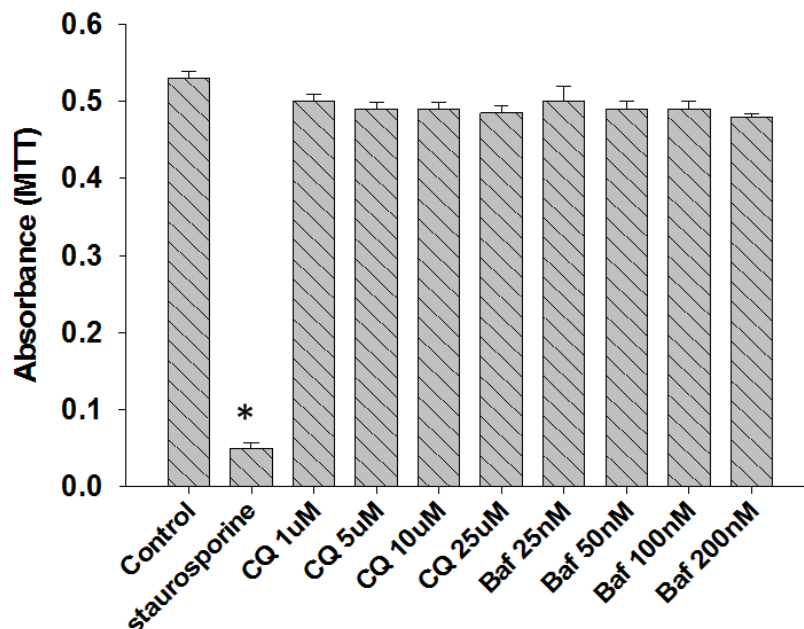


Figure 4.16. Dose response with autophagy inhibitors Chloroquine and Bafilomycin A1. MTT assay to assess cell toxicity of autophagy inhibitors, chloroquine and bafilomycin in H460 cells after 48 hours. Staurosporine (1 μ M) used as a positive control. Neither of the inhibitors were found to be toxic to cells (n=3, mean \pm SE, *p<0.05, compared to control).

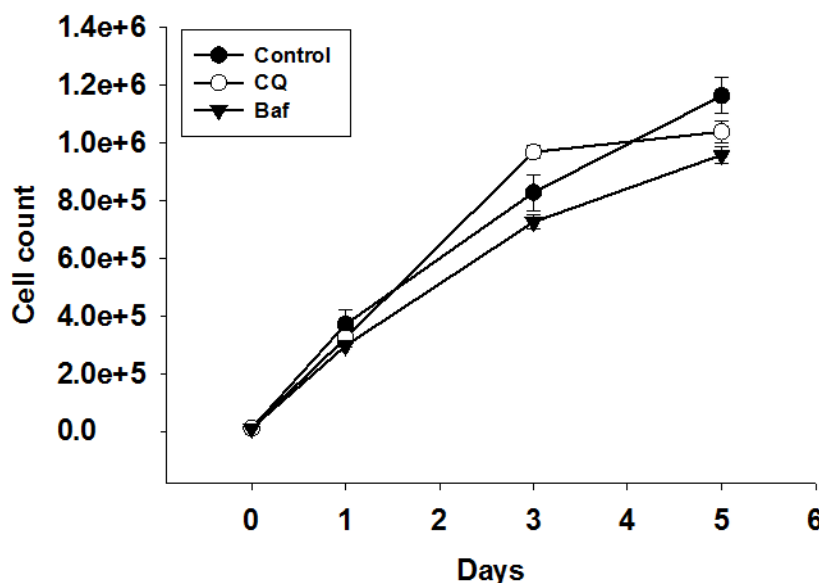


Figure 4.17. Minimal effect of autophagic inhibitors on cell viability. Cell viability studies were performed in H460 cells to assess growth inhibitory effects of autophagy inhibitors, chloroquine and bafilomycin at 10 μ m and 100 nM concentrations, respectively. Both inhibitors showed minimal inhibitory effects on cell viability.

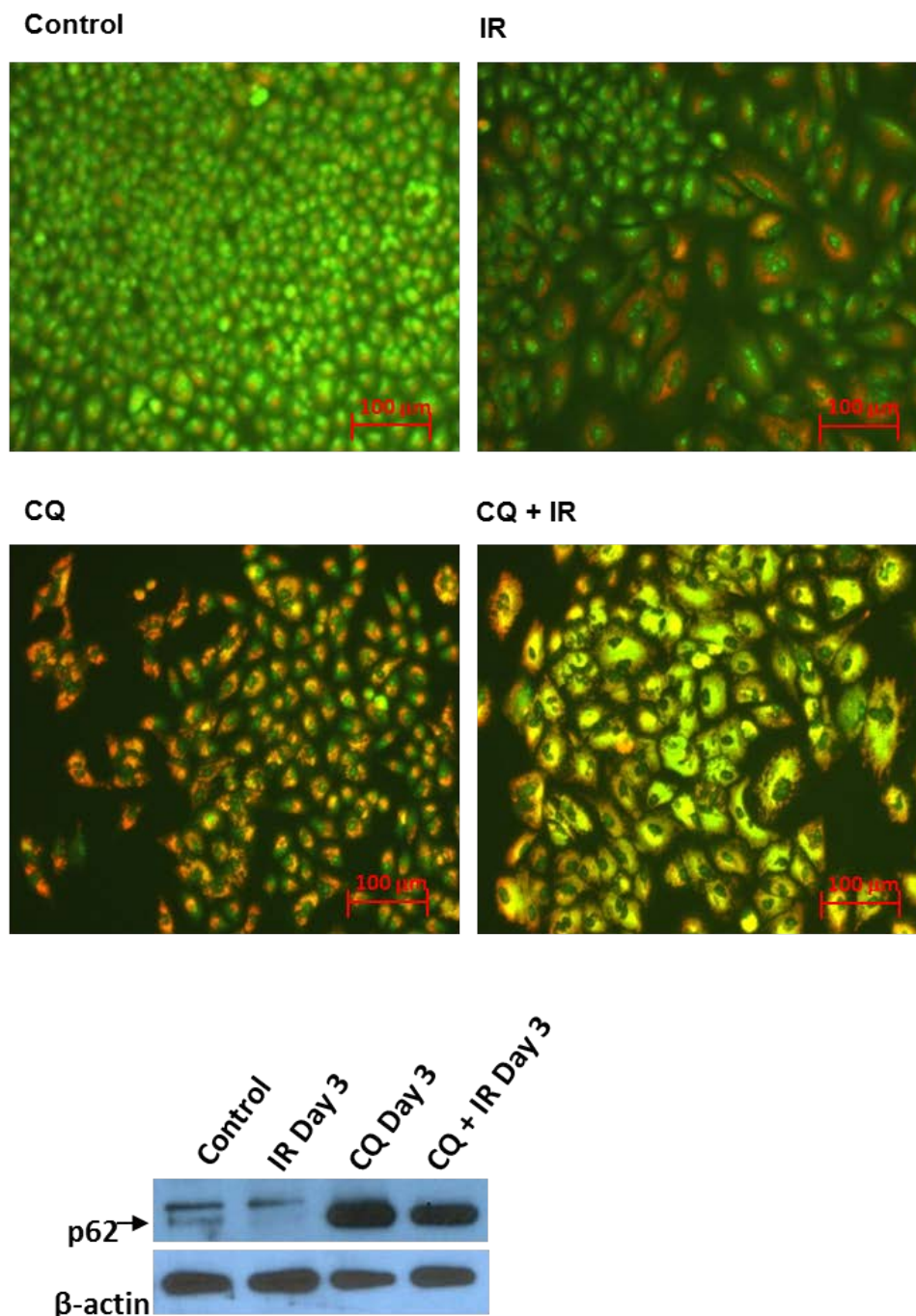


Figure 4.18. Inhibition of radiation induced autophagy in A549 cells. Acridine orange staining indicating autophagy inhibition by chloroquine (upper panel; all images taken at same magnification on day 3). Chloroquine interferes with the acidification step and turns orange color of vesicles to yellow. Western blotting indicating autophagy inhibition by accumulation of autophagic flux marker p62 in the presence of chloroquine on Day3 (lower panel).

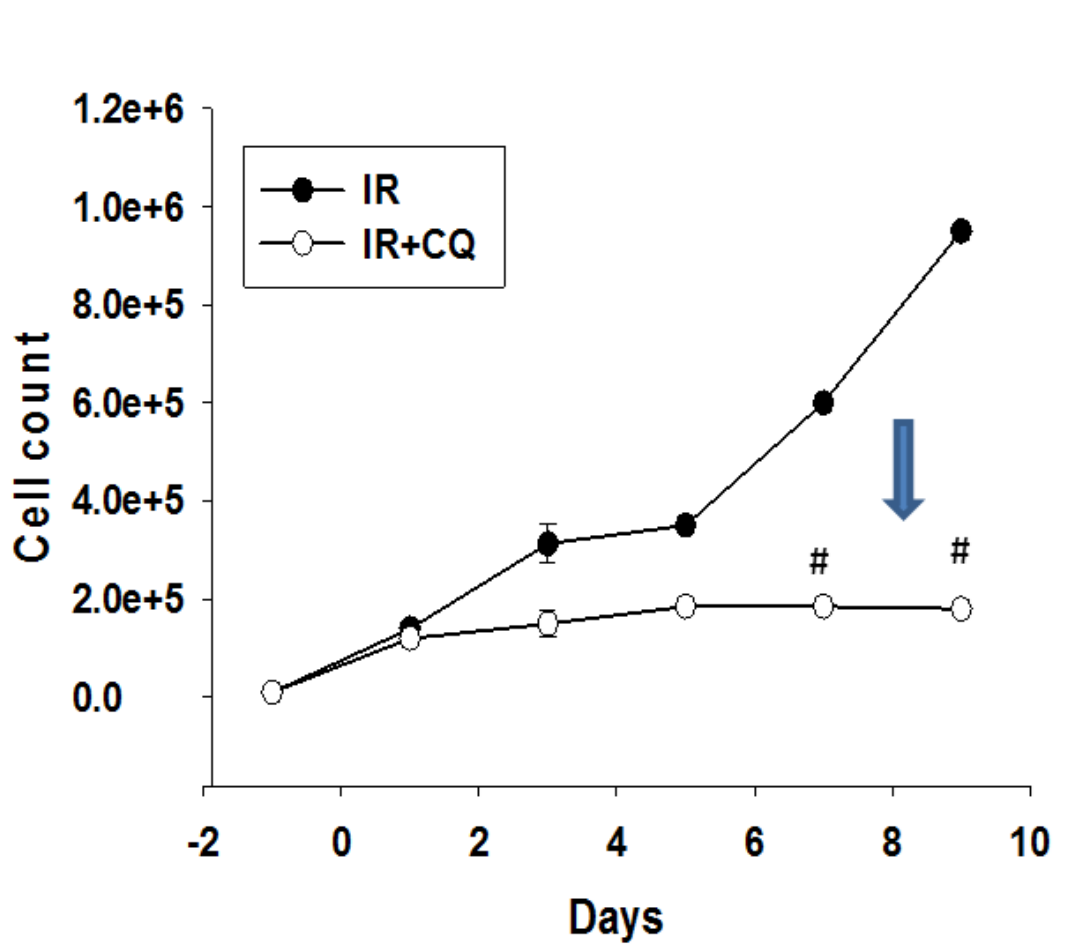


Figure 4.19. Cytoprotective autophagy induced by radiation in A549 cells. Evidence that autophagy induced by radiation (IR, 6 Gy) alone is cytoprotective in A549 cells based on increase in radiation sensitivity when autophagy is blocked by chloroquine (n=3, mean \pm SE, #p<0.001). These studies were carried out for a time period of 9 days.

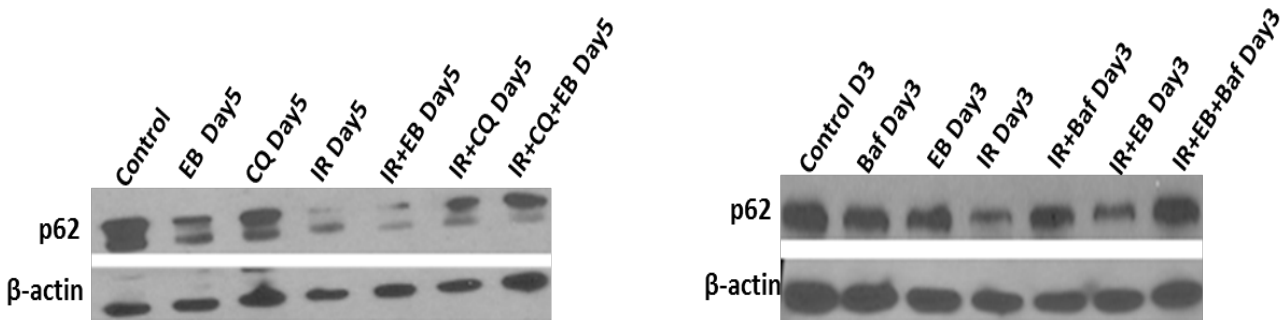
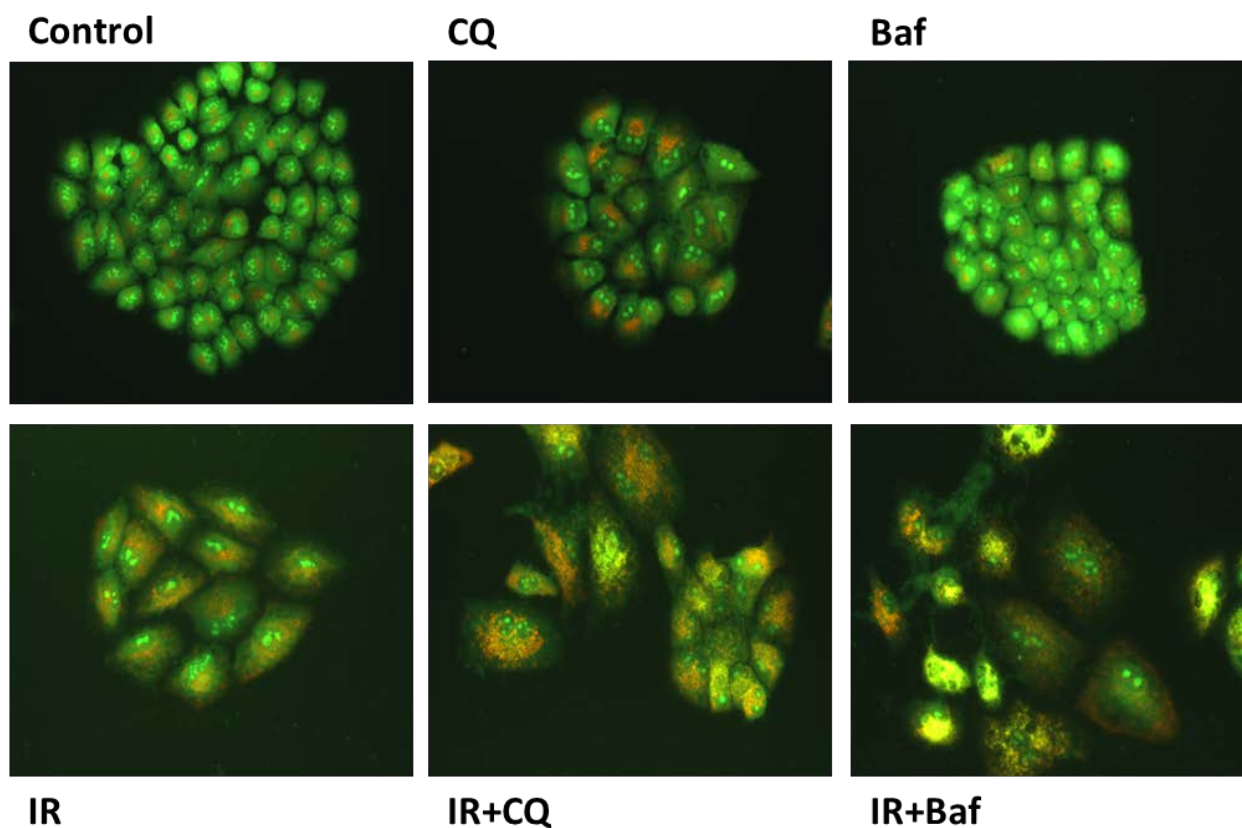


Figure 4.20. Inhibition of radiation induced autophagy in H460 cells. Acridine orange staining (upper panel) indicate autophagy inhibition with use of chloroquine and bafilomycin A1. Again, both chloroquine and bafilomycin A1 interfere with the acidification step and further turn the orange color of the vesicles to yellow. (all images were taken at the same magnification on day 3). Western blotting (lower panel) indicating autophagy inhibition by accumulation of autophagic flux marker p62 in presence of chloroquine and bafilomycin.

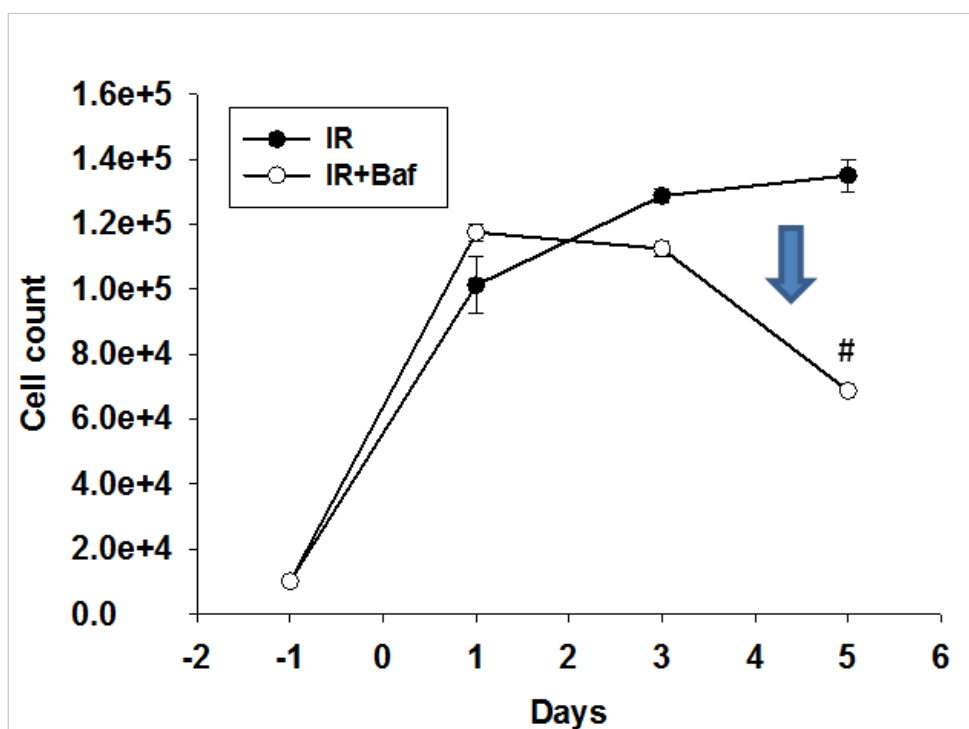
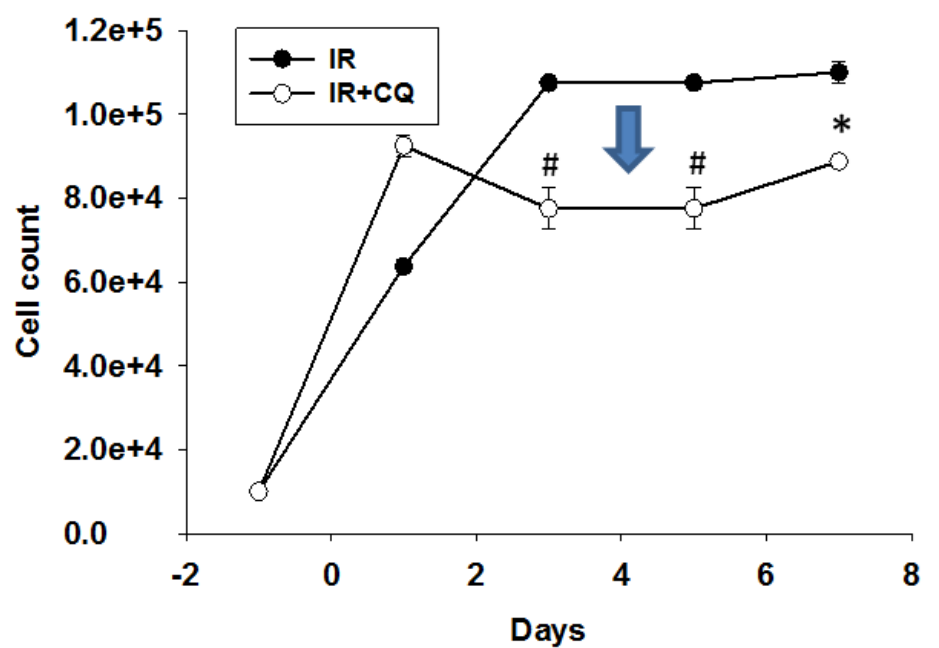


Figure 4.21. Cytoprotective autophagy induced by radiation in H460 cells. Evidence that autophagy induced by radiation (IR, 6 Gy) alone is cytoprotective in H460 cells based on increase in radiation sensitivity when autophagy is blocked by pharmacological inhibitors chloroquine (upper panel) and bafilomycin (lower panel) (n=3, mean \pm SE, *p<0.05; #p<0.001). Studies were carried out for a time period of 5 and 7 days.

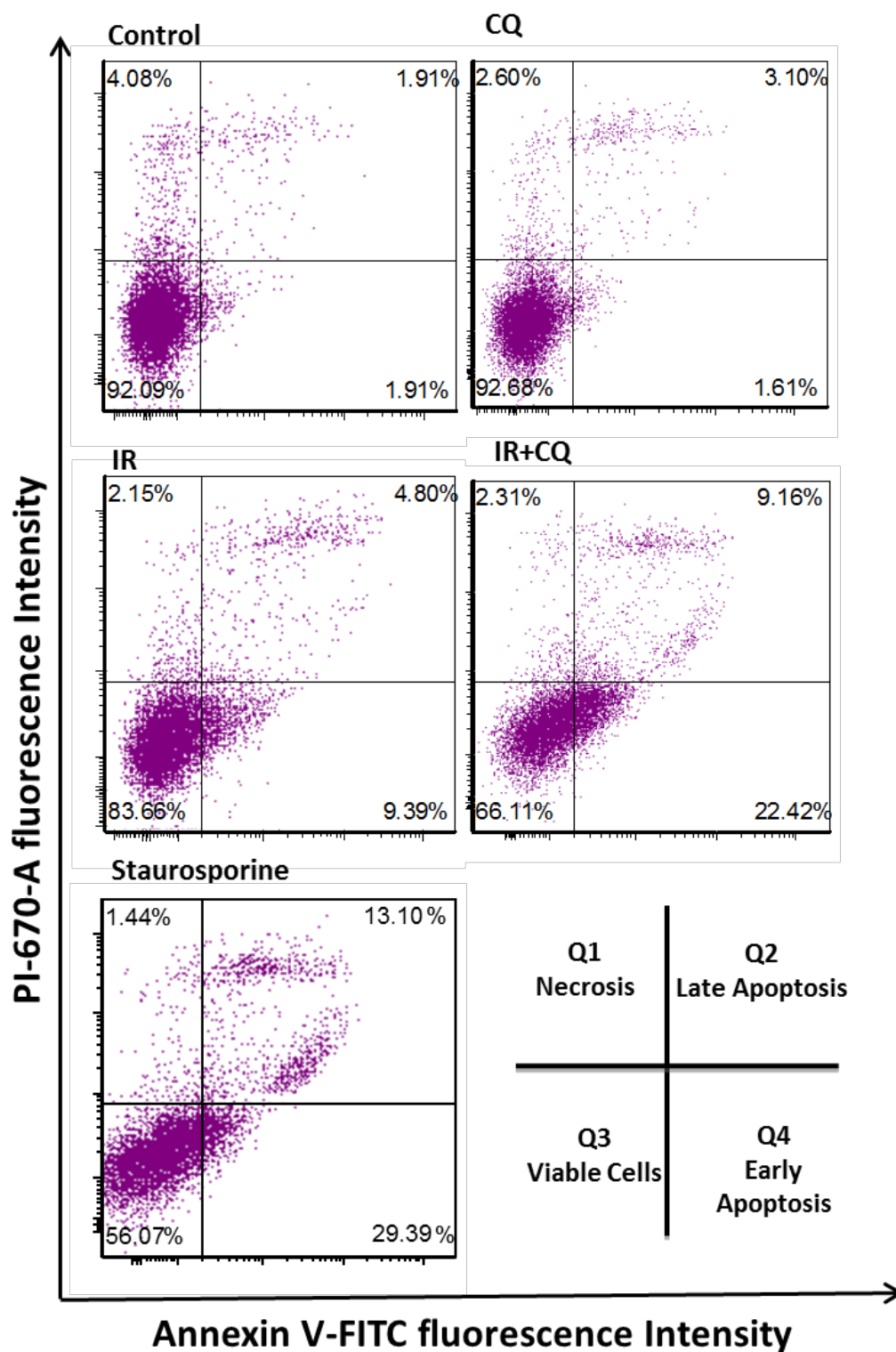


Figure 4.22. Assessment of apoptosis upon inhibition of autophagy in A549 cells by flow cytometry. Quantification of apoptosis by flow cytometry using annexin V/PI staining on day 3. These studies indicate an increase in apoptosis upon inhibition of radiation induced autophagy in A549 cells. Staurosporine used as a positive control.

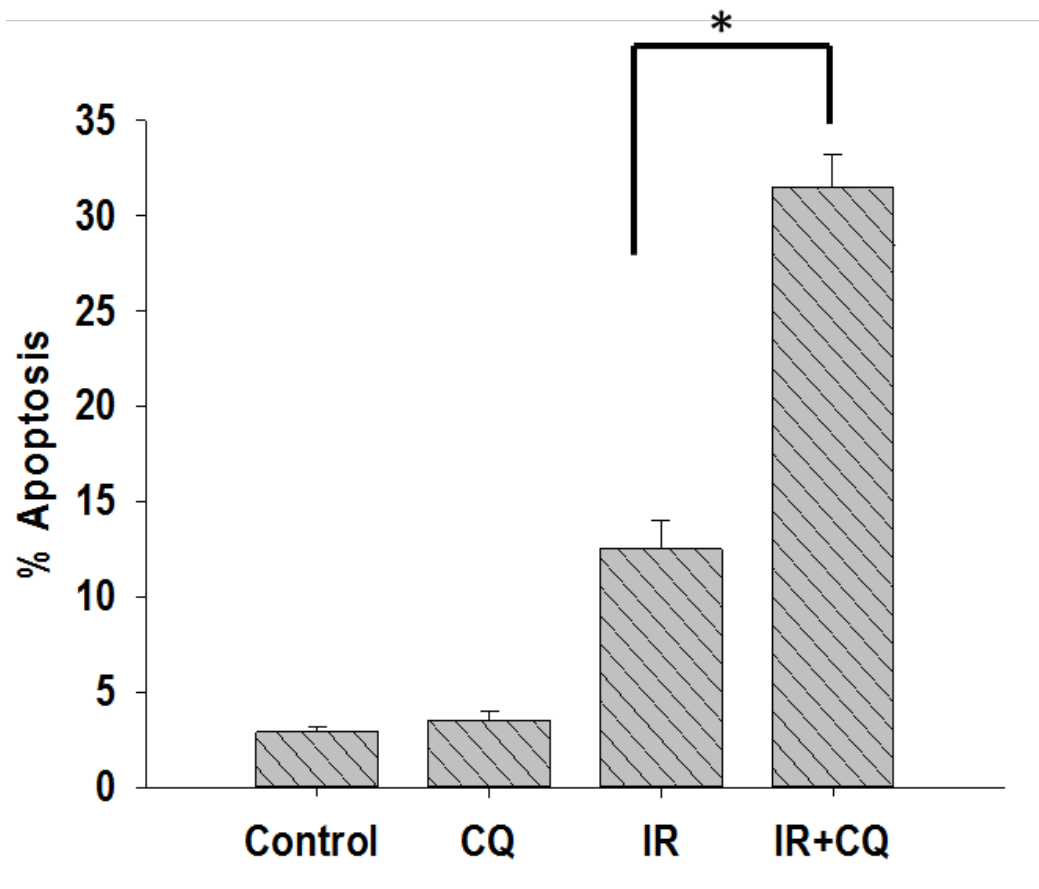


Figure 4.23. Induction of apoptosis upon inhibition of autophagy in A549 cells Measure of apoptosis when radiation induced autophagy is blocked using chloroquine in A549 cells (n=3, mean \pm SE, *p<0.05). induction in apoptosis levels is observed on day 3 when autophagy is inhibited using chloroquine (10 μ M).

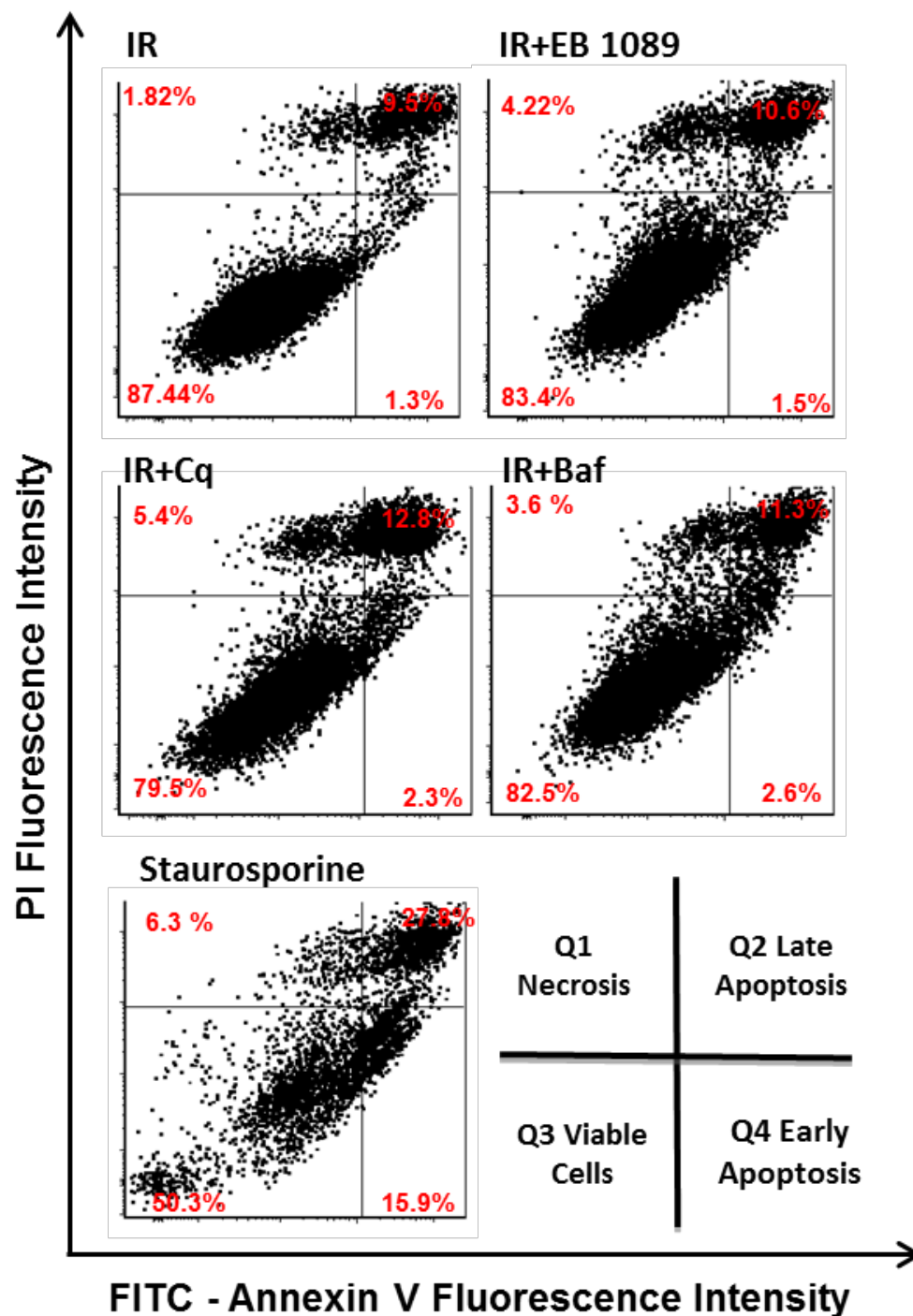


Figure 4.24. Assessment of apoptosis upon inhibition of autophagy in H460 cells by flow cytometry. Quantification of apoptosis by flow cytometry using annexin V/PI staining on day 3. These studies indicate the increase in apoptosis upon inhibition of radiation induced autophagy is not statistically significant in H460 cells. Staurosporine used as a positive control.

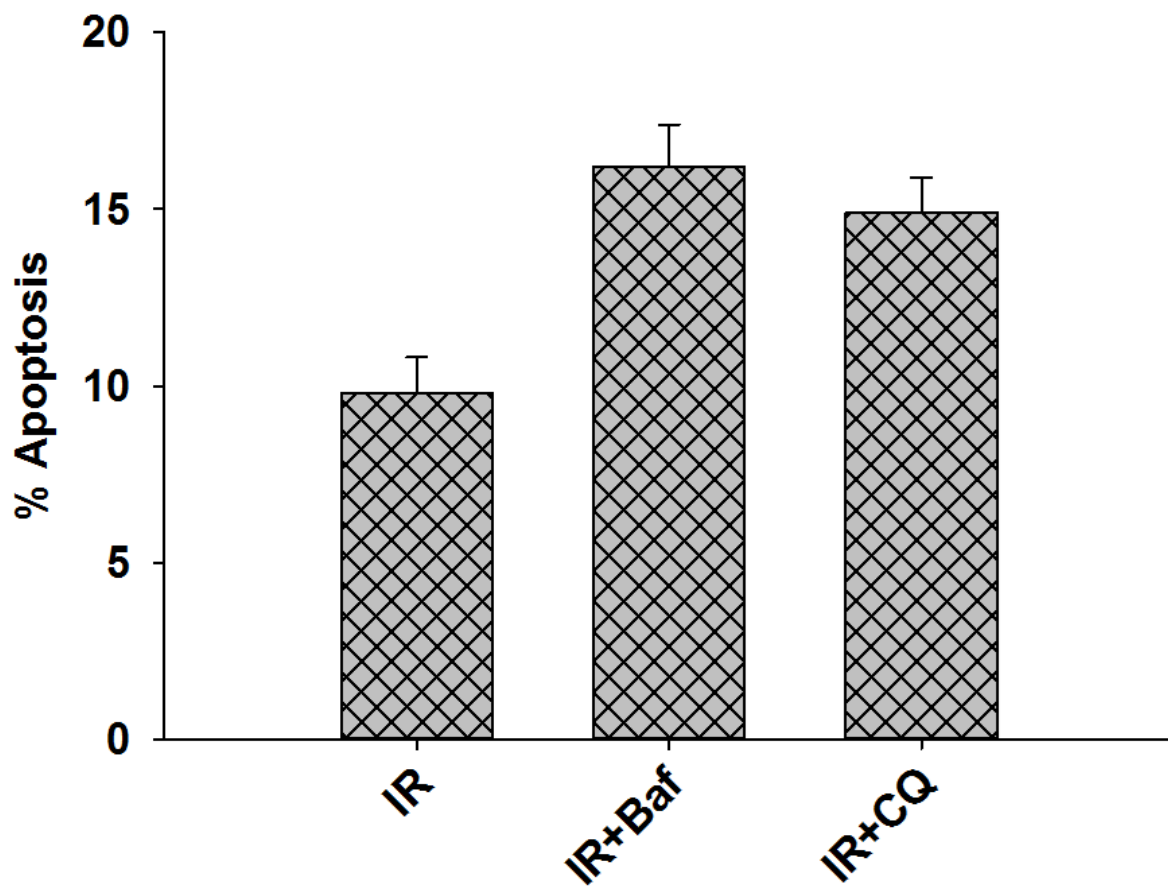


Figure 4.25. Induction of apoptosis upon inhibition of autophagy in H460 cells. Measure of apoptosis when radiation induced autophagy is blocked using bafilomycin A1(10 μ M) and chloroquine (100 nM) in H460 cells on day 3 (n=3, mean \pm SE). We did not observe a statistically significant increase in apoptosis upon autophagy inhibition with either of the inhibitors.

4.6. Increased sensitivity to the combination treatment of IR + EB 1089 is associated with a novel form of autophagy

To substantiate if autophagy is the mode of radiosensitization in response to the combination treatment of IR and EB 1089, we first determined whether interference with autophagy could reverse the impact of radiation + EB 1089. Again, we employed chloroquine and bafilomycin, both well-characterized pharmacological inhibitors of the late stages of autophagy (Klionsky DJ, *et al.* 2012). In addition, an shRNA strategy was used to knock down autophagy related genes such as *ATG5* and *BECN1*. *ATG5* is necessary for autophagy due to its involvement in autophagosome elongation (Mizushima N, *et al.* 2007). *BECN1* is a mammalian ortholog of the yeast autophagy gene *Atg6* that binds to class III phosphoinositide 3-kinase (PI3K) and is required for initiation of the formation of the autophagosome (Mizushima N, *et al.* 2007).

Acridine orange images shown in figure 4.26 confirm that autophagy was blocked by using pharmacological inhibitors in combination with both IR and IR+EB 1089. As mentioned before, both being late stage inhibitors lead to an increase in accumulation of acidic vesicles. Furthermore, figure 4.20 (lower panel) shows Western blots indicating that both inhibitors CQ and bafilomycin by themselves and in combination with radiation and EB1089, lead to an accumulation of p62, indicative of the inhibition of autophagic flux (Klionsky DJ, *et al.* 2012).

As shown in Figure 4.27, cell viability studies demonstrate that both chloroquine and bafilomycin, when used to block autophagy, reversed the radiosensitization effects of EB1089. This indicates that what we have termed a cytostatic form of autophagy (based

on the temporal response studies presented in Figure 3.7) is likely to be the mode of radiosensitization with the combination treatment. This conclusion is strongly supported by complementary genetic silencing studies, where the autophagy related genes *BECN1* and *ATG5* were knocked down utilizing shRNA as shown in the immunoblots in figure 4.28. Acridine orange images show a reduction in acidic vesicle formation confirming inhibition of autophagy as shown in figure 4.29. Cell viability studies shown in figure 4.30 after genetic silencing of *BECN1* and *ATG5* corroborate with the results of the pharmacological inhibition experiments. ***These studies further support our hypothesis that exposure of the NSCLC cells to 1,25 D₃ or EB 1089 could be altering the function of the autophagy induced by radiation, converting the cytoprotective autophagy to what we have termed cytostatic autophagy as indicated in figure 4.31 and 4.32.***

We further performed annexin V/PI staining assay to assess apoptosis when autophagy was inhibited. As indicated in figure 4.33 blocking autophagy did not significantly alter the level of apoptosis in response to the combination treatment of IR and EB 1089. However we do see an increase in apoptosis levels when radiation induced autophagy is inhibited which is expected due to the known cytoprotective nature of autophagy.

It is important to note that the sensitization to radiation that occurs when the cytoprotective autophagy induced by radiation is inhibited (Figure 4.12) is no longer evident in the cells exposed to EB 1089 + radiation; otherwise, it would be difficult to reverse the sensitization induced by EB 1089, a problem we encountered in our studies in breast tumor cells (Wilson EN, *et al.* 2011; Bristol ML, *et al.* 2012). Where, inhibiting autophagy induced by the combination treatment sensitized the cells since IR induced

autophagy was getting inhibited as well. This is the basis for our conclusion that the *cytoprotective* form of autophagy induced by radiation has been *converted* to a *cytostatic* form as shown in figures 4.29 and 4.30.

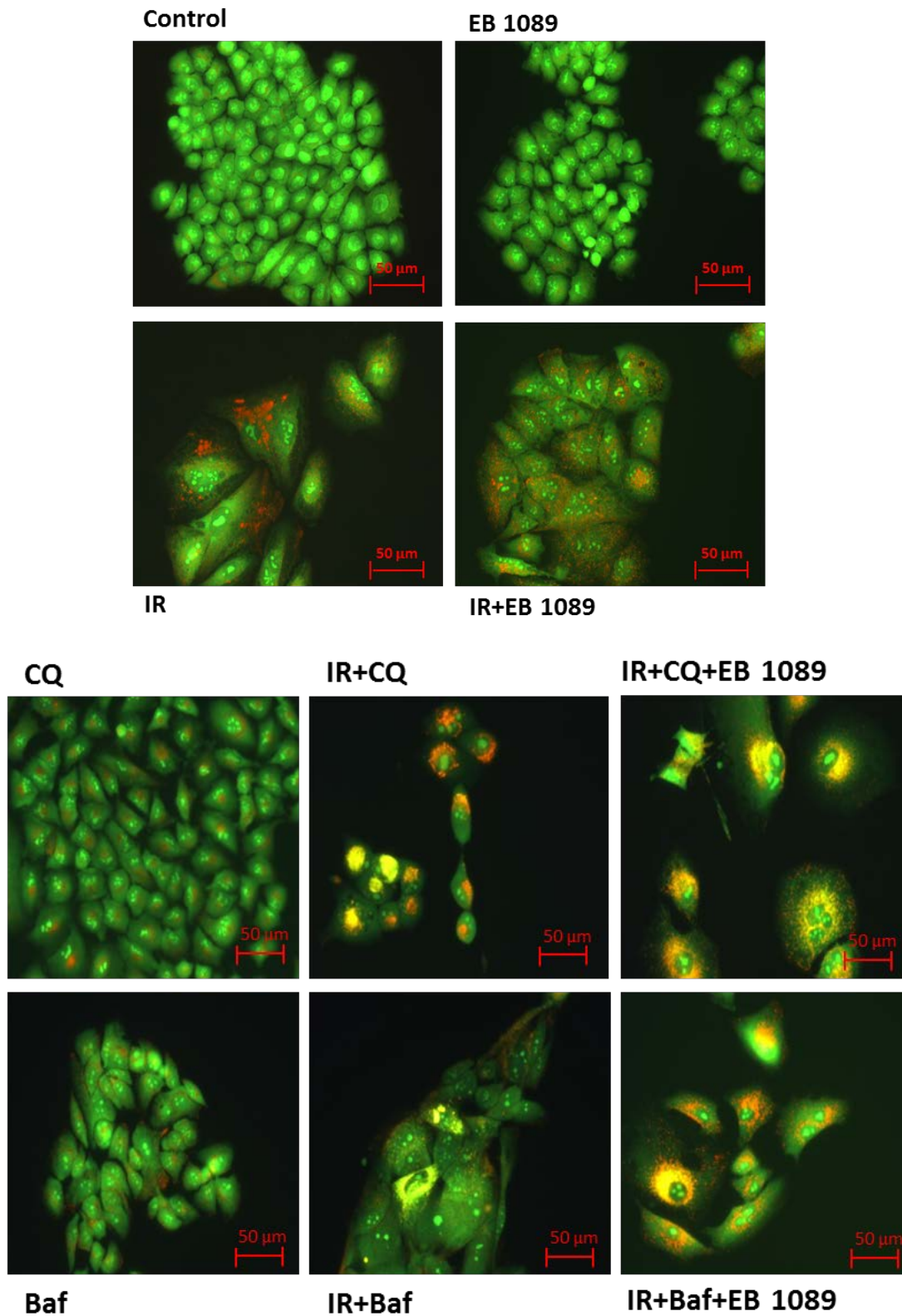


Figure 4.26. Evidence for inhibition of autophagy by chloroquine and bafilomycin A1 in H460 cells. Inhibition of autophagy indicated by increased acidic vesicle formation on use of late stage autophagy inhibitors, chloroquine (10 μ M) or bafilomycin (100 nM) in combination with IR and IR+ EB1089 in H460 cells on day 3 (all images were taken at the same magnification). Both inhibitors interfere with the acidification step in the autophagy process and further turns the orange color of the vesicles to yellow.

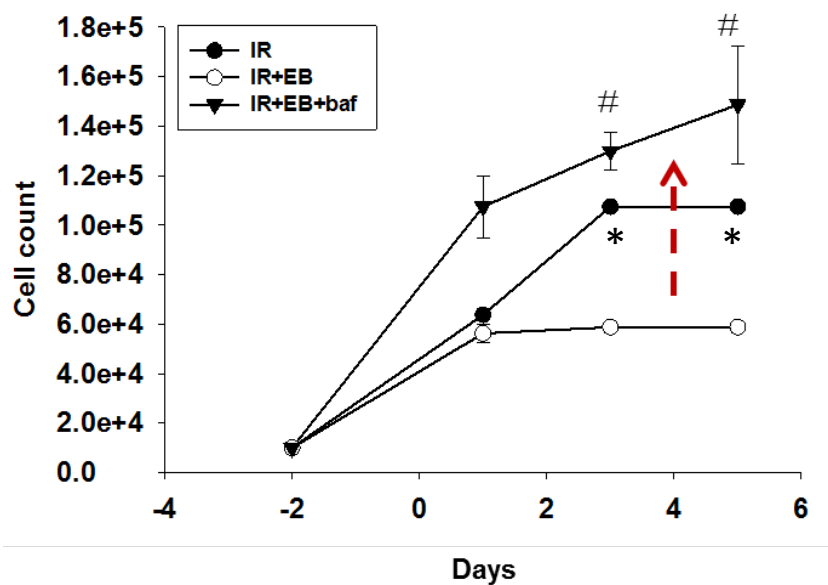
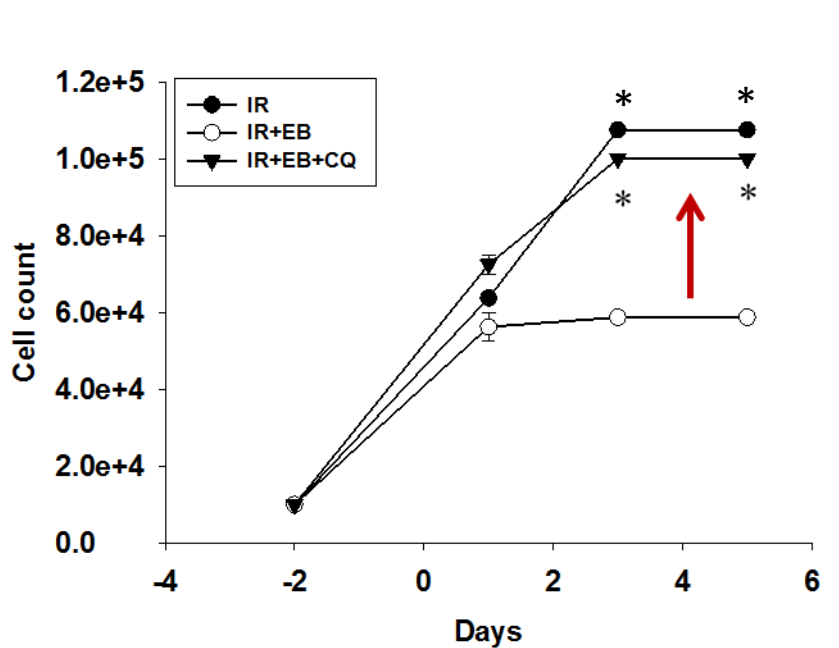


Figure 4.27. Evidence that radiosensitization by EB 1089 occurs through a cytostatic function of autophagy. Cell viability studies were conducted with autophagy inhibitors, chloroquine (CQ, 10 μ M; upper panel) or bafilomycin A1 (baf, 100 nM; lower panel) for a period of 5 days in h460 cells. (# p <0.001; * p <0.05; n =3, mean \pm SE). Reversal in sensitization is observed when autophagy is blocked using either chloroquine or bafilomycin.

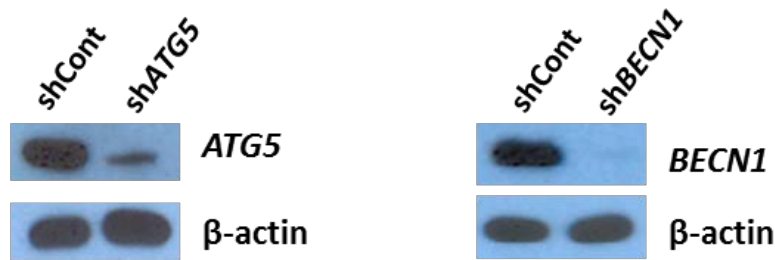


Figure 4.28. Knockdown of autophagy related genes, *ATG5* and *BECN1*, in H460 cells. Western blotting indicating knockdown of autophagy related genes, *ATG5* and *BECN1*, in H460 cells.

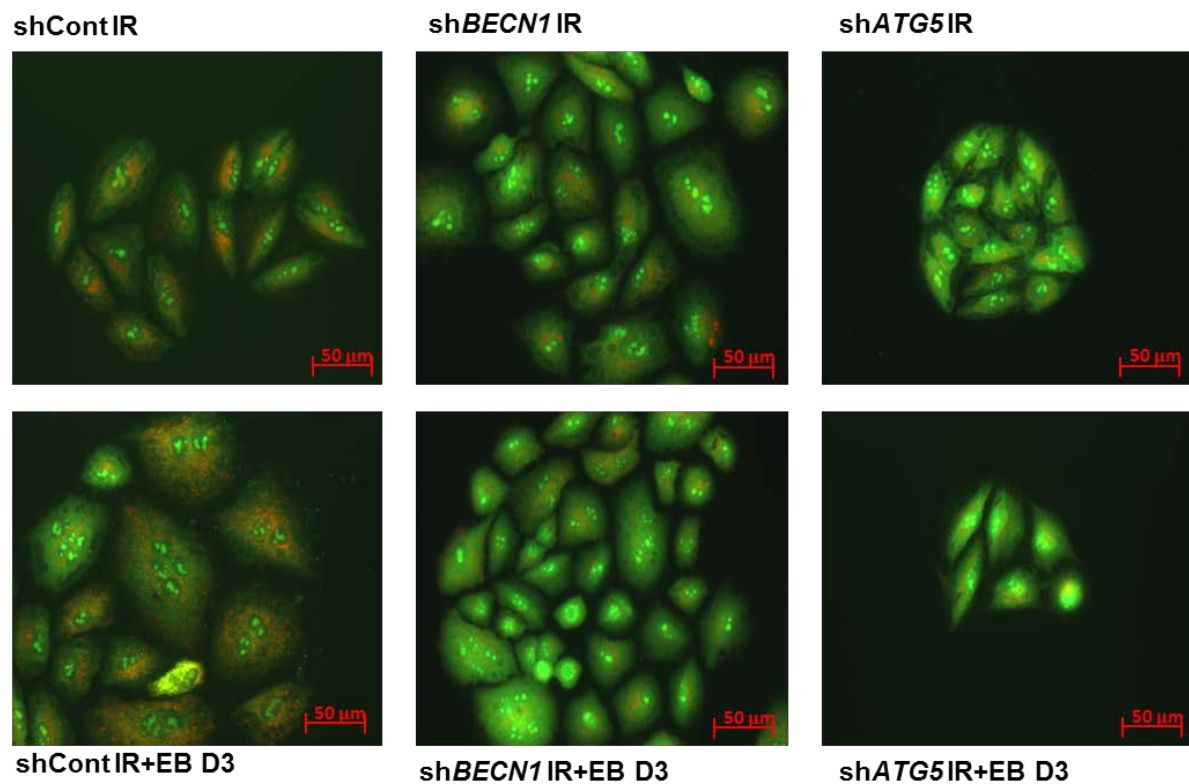


Figure 4.29. Inhibition of autophagy by genetic silencing of *ATG5* and *BECN1* in H460 cells. Decreased acidic vesicle formation when compared to control IR and IR+ EB 1089 treated samples, indicate autophagy inhibition in H460 sh*BECN1* and sh*ATG5* cells on day 3 (lower panel) (all images were taken at the same magnification).

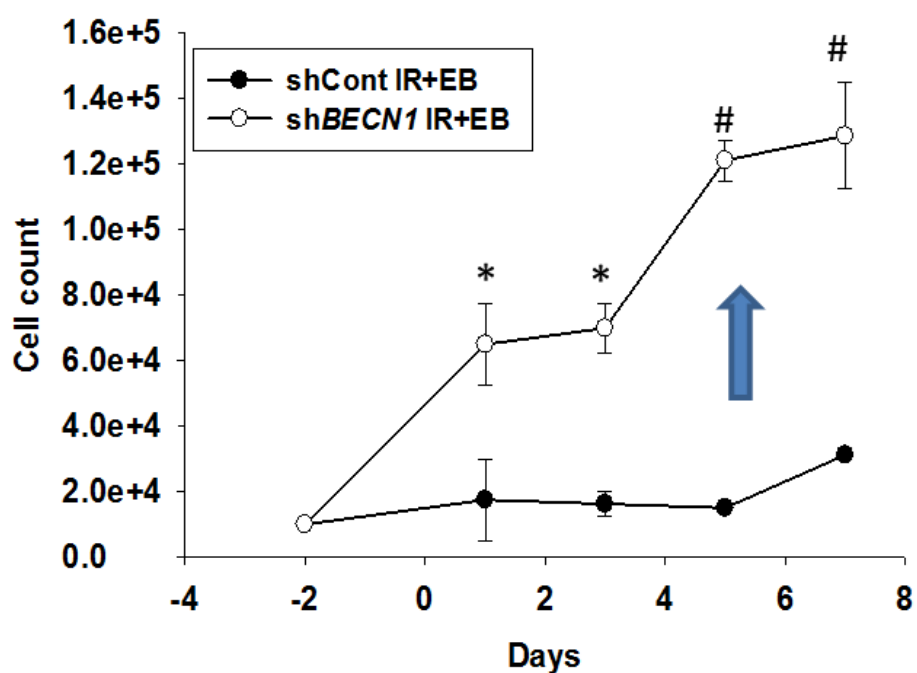
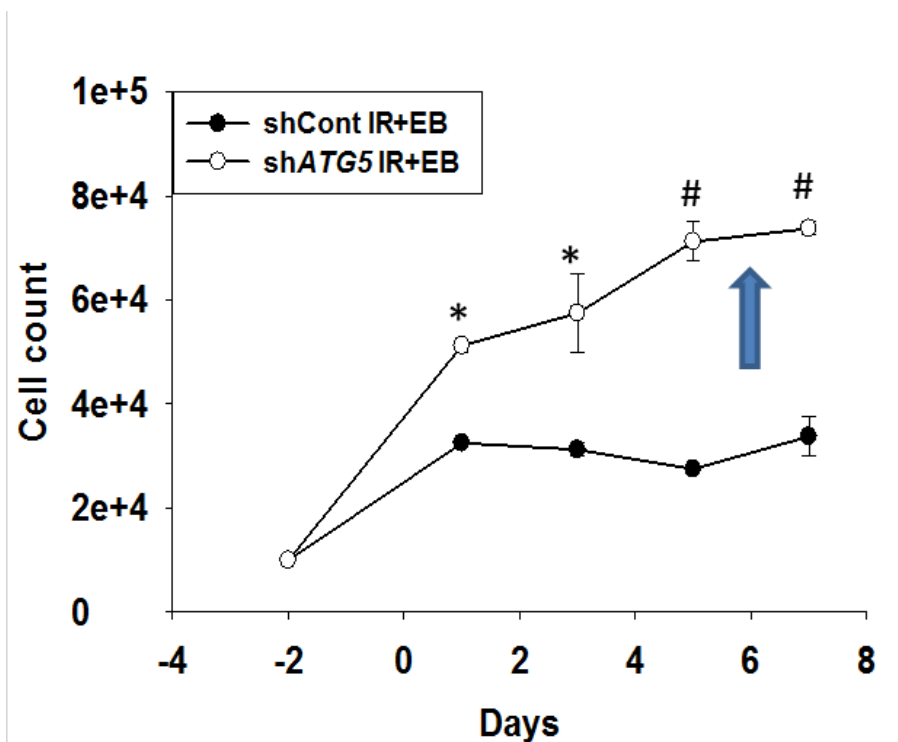


Figure 4.30. Genetic knockdown of *ATG5* and *BECN1* reverses EB 1089 induced radiosensitization. Cell viability studies were conducted to assess the effects of *ATG5* and *BECN1* knockdown in H460 cells. Increase in cell count after genetic knockdown of *ATG5* and *BECN1* indicates reversal in radiosensitization (n=3, mean \pm SE, *p<0.05, #p<0.001).

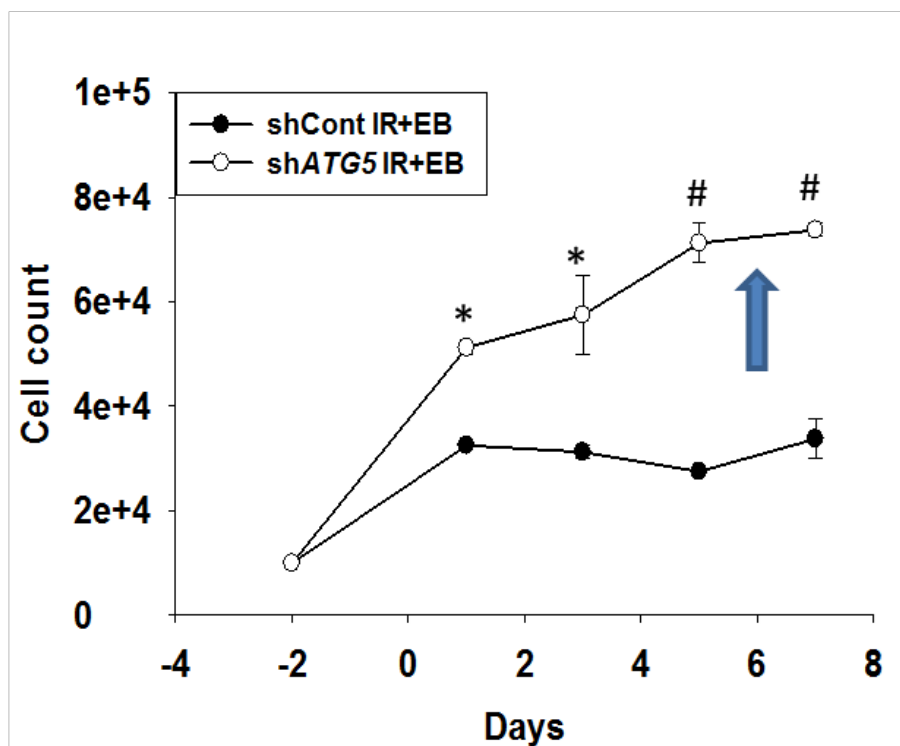
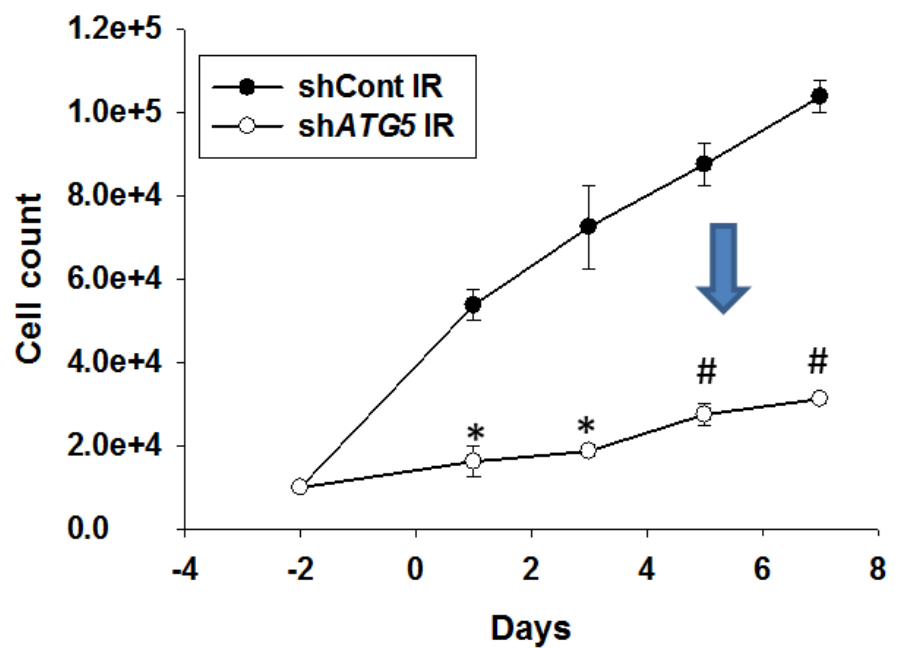


Figure 4.31. EB 1089 causes a change in function of autophagy as indicated upon autophagy inhibition by knockdown of *ATG5* gene. Change from cytoprotective function (in response to radiation) to cytostatic function (in response to the combination of IR+EB 1089) evident upon inhibition of autophagy by knocking down autophagy related gene *ATG5*. Cell viability studies were carried out for a time period of 7 days (n=3, mean \pm SE, *p<0.05, #p<0.001).

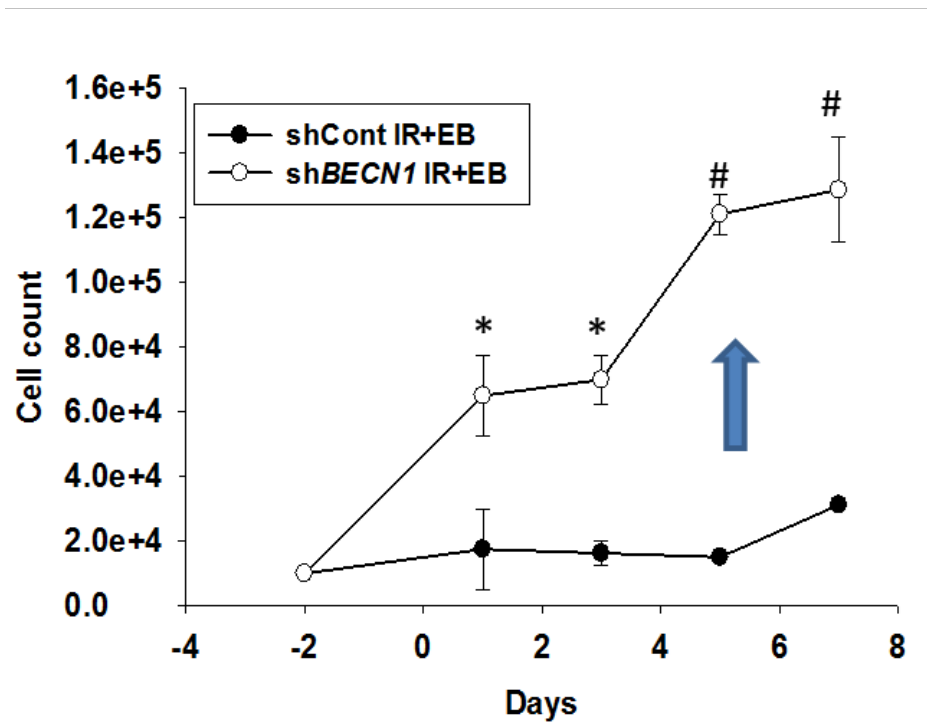
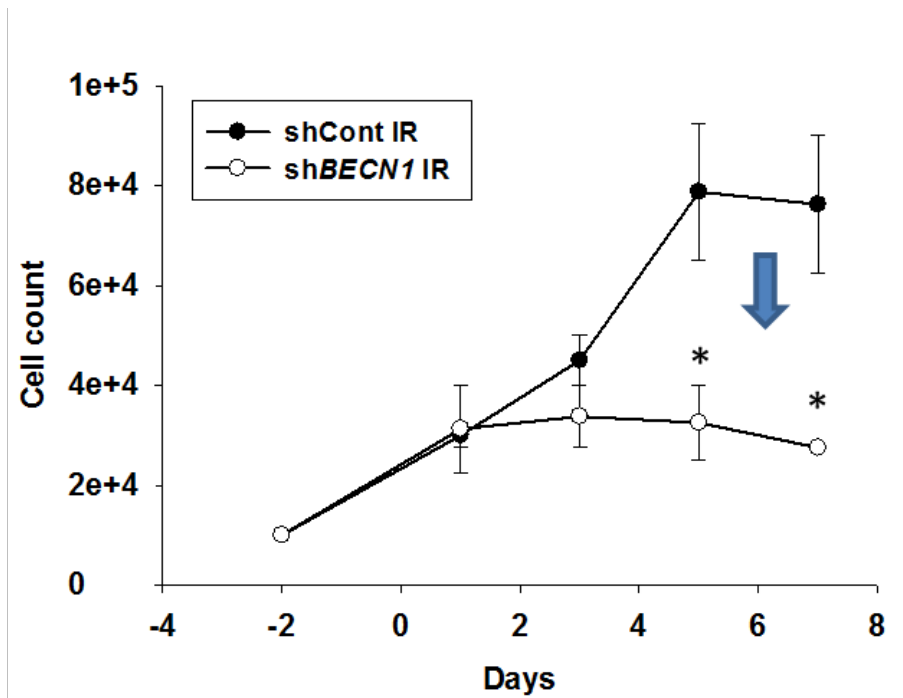


Figure 4.32. EB 1089 causes a change in function of autophagy as indicated upon autophagy inhibition by knockdown of *BECN1* gene. Change from cytoprotective function (in response to radiation) to cytostatic function (in response to the combination of IR+EB 1089) evident upon inhibition of autophagy by knocking down autophagy related gene *BECN1*. Cell viability studies were carried out for a time period of 7 days (n=3, mean \pm SE, *p<0.05, #p<0.001).

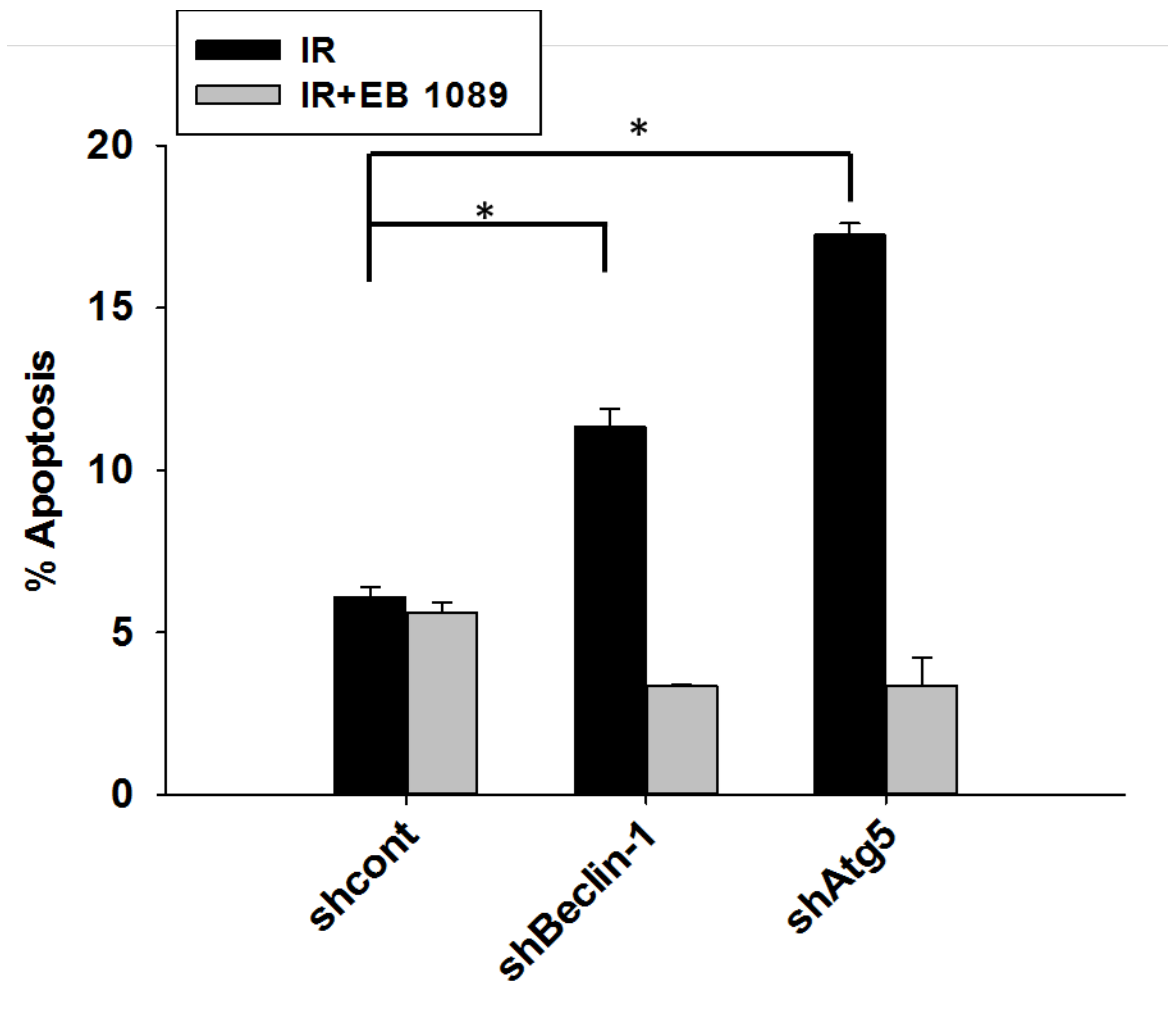


Figure 4.33. Influence of genetic silencing of autophagy on apoptosis induction by IR and IR+EB 1089 in H460 cells. Apoptosis induction upon autophagy inhibition assessed by flow cytometry on day 3. Annexin V/ PI staining was done to assess both early and late apoptosis. (n=3, mean \pm SE; *p<0.05). Upon inhibition of autophagy by knockdown of ATG5 and BECN1, there was an increase in apoptosis in response to radiation and response to IR+EB 1089 remained unchanged.

4.7. Effects of radiation and EB 1089 + radiation are associated with cell cycle arrest

As shown in the temporal response studies presented in Figure 3.7, the primary effects of radiation as well as radiation in combination with either 1,25-D₃ or EB 1089 were to arrest cell growth without evidence for cell killing in either condition. To confirm these observations, cell cycle analysis was performed in H460 cells exposed to radiation alone and EB 1089 + radiation with and without knock down of the autophagy genes *ATG5* and *BECN1*. As depicted in Figure 4.34, radiation alone as well as EB 1089 + radiation reduced the S phase population and increased the G2/M phase population. Effects of EB 1089 alone were not significant, again consistent with its lack of effect on cell growth. As would be expected, inhibition of autophagy by genetic silencing reversed the G2/M associated growth arrest.

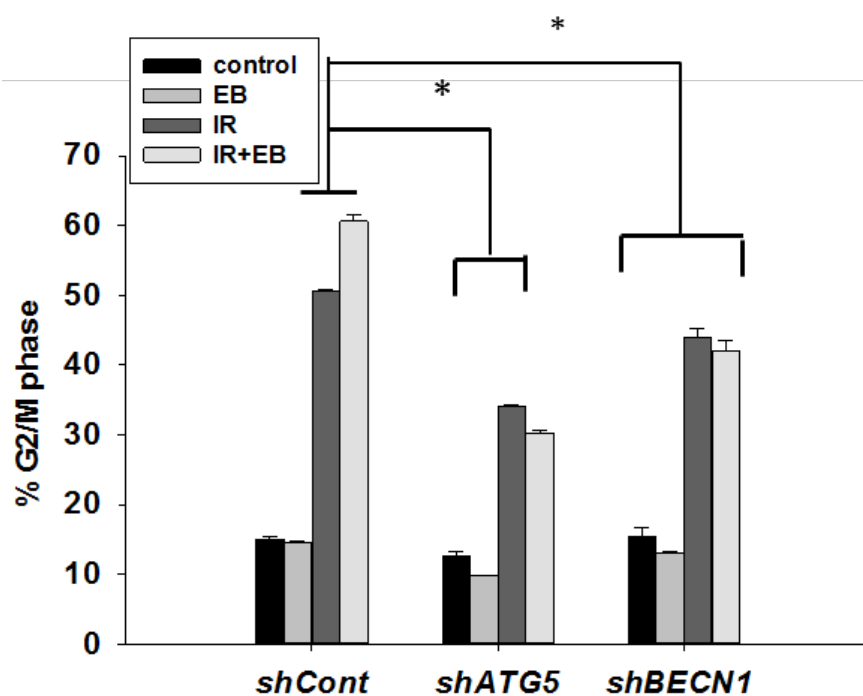
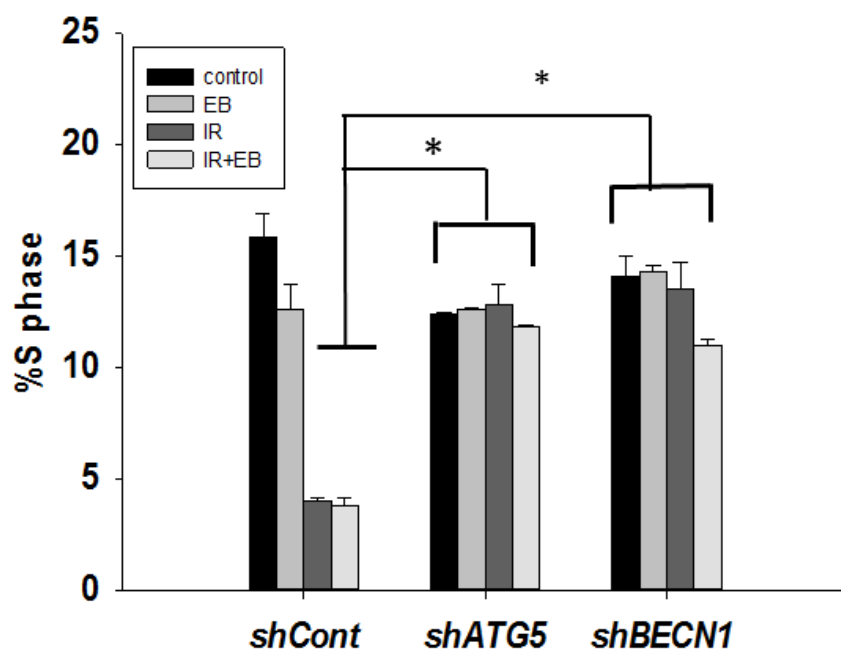


Figure 4.34. Cell Cycle analysis. Cell cycle analysis was performed in H460 (*shcont*, *shATG5*, and *shBECN1*) cells. Percentage of cells undergoing S-phase is shown in upper panel (n=2, mean \pm SE, *p<0.05). Percentage of cells arresting in G2/M phase is shown in lower panel. (n=2, mean \pm SE, *p<0.05).

4.8. Effects of 1,25-D₃ and EB 1089 on radiation sensitivity in normal cells.

Drug selectivity is a critical consideration in cancer related studies; treatments should ideally be highly targeted to minimize the damage to normal cells and tissue. This is particularly true for the utilization of radiation in diseases such as non-small cell lung cancer. Consequently, studies were conducted to test for selectivity of the combination treatment of 1,25-D₃ with radiation in normal human bronchial epithelial cells and cardiomyocytes in culture. As with the experiments in H460 cells, the HBEC3-KT bronchial epithelial cells were pretreated with 100 nM EB 1089 24hours before irradiation with a single dose of 6Gy while the H9C2 cardiomyocytes were exposed to 100nM 1,25-D₃ for 24 hours followed by a single dose of radiation (5 Gy). Figures 4.34 and 4.35 indicate that there were no significant differences between the impact of radiation alone and the combination of radiation + 1,25-D₃ or radiation + EB 1089 treatments in the epithelial cells or the cardiomyocytes. This suggests that if we can make radiation treatment focused and limit it to specific tumor site, we can use the combination treatment in clinics since vitamin D by itself does not have any toxic effects.

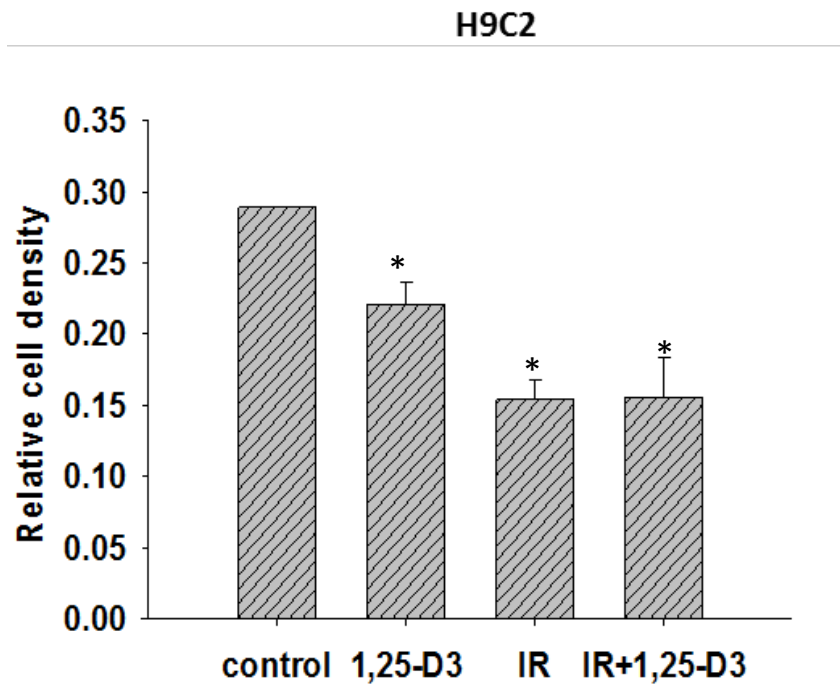


Figure 4.35. No difference in sensitization by IR compared to IR+1,25-D₃ in cardiomyocytes. MTT assay conducted in H9C2 cardiomyocytes after exposure to 100nM 1,25-D₃ for 24 hours followed by radiation. (5 Gy). (n=3, mean ± SE). No significant differences between treatments of IR and the combination of IR+ 1,25-D₃ were observed. 1,25-D₃ by itself did not have any inhibitory effects on the growth of cells.

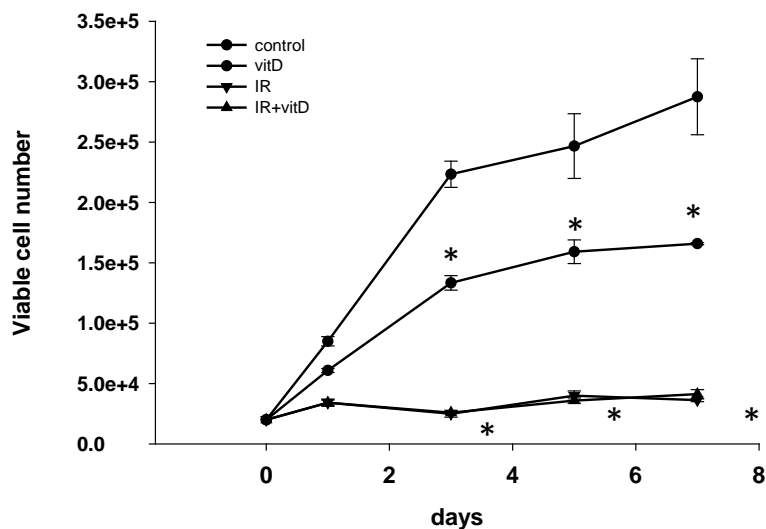


Figure 4.36. No difference in sensitization by IR compared to IR+1,25-D₃ in human bronchial epithelial cells. Cell viability studies were conducted in Human bronchial epithelial cells after exposure to 100nM 1,25D₃, radiation (IR, 6Gy), or 100nM 1,25D₃ prior to irradiation (6 Gy). 1,25-D₃ by itself showed moderate inhibitory effects on the growth of cells. No significant differences between treatments of IR and the combination of IR+ 1,25-D₃ were observed (n=3, mean ± SE, *p<0.05 compared to control).

4.9. Summary.

Initially, data pertaining to autophagy induction produced in A549 cells indicated that an increase in autophagy could be the mode of sensitization in response to the combination treatment in NSCLC. This conclusion was further supported by data demonstrating lack of other modes of sensitization such as DNA damage, apoptosis, and senescence. A number of studies used to assess autophagy indicated that there was a significant increase in autophagy levels in response to both IR and the combination treatment of IR + 1,25-D3/EB 1089. However, studies conducted in H460 made a strong case that an increase in autophagy levels was not responsible for the sensitization but that there had been a switch in autophagic function.

Pharmacological and genetic inhibition studies indicated that the functions of autophagy produced in response to radiation and IR + EB 1089 were different in nature. Inhibition of radiation induced autophagy led to an increase in sensitization, supporting the premise of cytoprotective autophagy being activated in response to radiation alone. This sensitization to radiation by autophagy inhibition appears to be a result, in part, from an increase in apoptosis. On the other hand inhibition of autophagy induced by the combination treatment led to a reversal in sensitization. ***These studies support our hypothesis that exposure of the NSCLC cells to 1,25 D₃ or EB 1089 could be altering the function of the autophagy induced by radiation, converting the cytoprotective autophagy to what we have termed cytostatic autophagy.***

Cell cycle studies performed with H460 cells showed growth arrest in response to both IR and the combination treatment of IR and EB 1089, which was reversed when

autophagy was inhibited by knockdown of autophagy related genes, *ATG5* and *BECN1*. Studies conducted with normal human epithelial cells and cardiomyocytes indicate the possibility of the combination treatment being clinically relevant since the growth inhibition resulting from the combination treatment is no greater than that caused by radiation alone.

Chapter 5

Exploring the signaling pathways involved in vitamin D mediated radio sensitization

The studies described in this chapter were designed to establish and understand signaling pathways involved in vitamin D mediated radio-sensitization and furthermore to distinguish the signaling pathways that might be involved in altering the different functions of autophagy.

5.1. p53's involvement in radio-sensitization by vitamin D.

In our previous work with breast tumor cells, we identified a fundamental requirement for functional p53 in radiosensitization by 1,25D₃/EB 1089 (Sundaram S, et al. 1999; Bristol ML, et al. 2012; Wilson EN, et al. 2011). Both A549 and H460 cells are known to express wild type p53 (Cheng G, et al. 2013; Lorin S, et al. 2013). We showed earlier

that the combination treatment of radiation +EB 1089 failed to increase sensitivity to radiation in the H358 NSCLC cell line (Figure 3.8), which has mutant p53, despite the very high expression of the VDR (Figure 3.1).

To confirm the requirement for functional p53 in radiosensitization, we employed H1299 cells which are p53 null (Zhang F, et al. 2008). H1299 p53 null cells were transfected in order to make them express p53 in the presence of doxycycline (1 µg/ml). The expression of p53 induced by doxycycline was confirmed by Western blotting as shown in figure 5.1. Further we demonstrate (Figure 5.2 A & B, where 5.2B is an expanded scale for IR and IR+EB 1089) that sensitization to radiation by EB 1089 fails to occur in the H1299 NSCLC cell line, which is p53 null, but expresses the VDR (Figure 3.1). In dramatic contrast, figures 5.3 A & B (5.3B is an expanded scale for IR and IR + EB 1089) indicate that H1299 cells could be radiosensitized by EB 1089 when p53 was expressed under the influence of doxycycline.

We performed acridine orange staining to confirm that the induction of autophagy occurred in both cell lines in response to the different treatment conditions. Both H1299 parental cells which are p53 null (-Dox) and H1299 cells with induced p53 (+Dox) showed a significant increase in acidic vesicle formation with both IR and the combination of IR+EB 1089 as shown in figures 5.4 and 5.5.

To confirm and extend these observations in the H1299 cells, we investigated the effects of both pharmacological and genetic autophagy inhibition in both the p53 null and inducible cell lines. We performed acridine orange staining to ensure that autophagy was being inhibited using chloroquine in H1299 p53 null (-Dox) and p53

induced (+Dox) cells. Figures 5.6 and 5.7 show an accumulation of acidic vesicles when chloroquine is used in combination with IR+EB 1089 in both H1299 (-Dox) and H1299 (+Dox) cells since it is a late stage autophagy inhibitor. Further, figure 5.8 shows that chloroquine effectively promoted accumulation of LC3II, which is indicative of interference with the late stage of autophagy in both p53 null (-Dox) and p53 inducible (+Dox) H1299 cells. We performed cell viability studies to assess the effect of pharmacological inhibition of autophagy on cell growth. Figure 5.9 indicates that in H1299 cells with induced p53 (+Dox), sensitization of the combination treatment was reversed when autophagy was blocked using chloroquine. In quite dramatic contrast, blocking autophagy in the p53 null H1299 parental cells (-Dox) had minimal effect on tumor cell sensitivity to the combination treatment of EB1089 + radiation (Figure 5.9).

For genetic silencing studies, as shown in figure 5.10, both Atg5 and Beclin-1 were successfully knocked down for p53 null (-Dox) and p53 inducible (+Dox) H1299 cells. Figures 5.11 and 5.12 show the reduction in autophagic vesicle formation when Atg5 and Beclin-1 were knocked down in both p53 null (-Dox) and p53 inducible (+Dox) H1299 cells. Similar to the pharmacological inhibition studies, genetic inhibition of autophagy in the p53 induced cell line (+Dox) led to a reversal in sensitization compared to H1299 p53/null (-Dox), where no change with autophagy inhibition was observed as shown in figures 5.13 and 5.14.

It should be noted that the low cell number for radiation alone in Figures 5.9B, 5.13B and 5.14B reflects sensitization to radiation through interference with the cytoprotective function of radiation-induced autophagy. Conversely, the high cell numbers for the EB

1089 + radiation combination reflects the fact that sensitization was eliminated through interference with cytosstatic autophagy.

5.2. The AMPK signaling pathway:

AMPK, an energy sensor protein kinase, has been shown to be involved in regulating autophagy (Hardie DG, et al. 2012; Inoki K, et al. 2012). Studies were performed to investigate changes in AMPK levels by measuring its phosphorylation at Threonine 172 site, in response to radiation alone (cytoprotective autophagy) and radiation + EB1089 (putatively cytosstatic autophagy).

Western blotting was employed to assess the changes in phosphorylation levels of AMPK and flow cytometry was used to quantitate and measure the percentage of phosphorylated proteins. All of these studies were performed at early time points, 4 and 12 hours. As indicated in the western blot shown in figure 5.15, we observed an increase in pAMPK levels as early as 4hrs in cells receiving the combination treatment of IR+EB1089 compared to cells receiving IR treatment alone. At both the 4 and 12 hour time points, AMPK activation by the combination treatment significantly exceeded that for radiation alone as indicated in figures 5.16.

To potentially distinguish between the involvement of AMPK in autophagy signaling by radiation alone and radiation + EB1089, compound C, a pharmacological inhibitor of AMPK was used (Yang WL, et al. 2012). Western blotting was performed to demonstrate that compound C was able to inhibit radiation and radiation+EB1089 induced AMPK phosphorylation as shown in figure 5.17. We also evaluated phosphorylation levels of Acetyl-CoA carboxylase (ACC), a downstream target of AMPK

(Vazquez-Martin A, et al. 2013). Inhibition of the phosphorylation of ACC further confirmed compound C's effect. Figure 5.18 indicates that compound C by itself did not have any effect on the viability of cells. Autophagy was monitored by assessing GFP-LC3 redistribution 12 hours post treatment by confocal microscopy as shown in figure 5.19. Compound C treatment significantly diminished LC3 redistribution in IR and IR+EB 1089 treated cells. Furthermore, figure 5.20 (lower panel) shows that inhibition of AMPK by compound C resulted in reversal of the radiosensitizing effects of EB 1089. In dramatic contrast, treatment with compound C *enhanced* the response to radiation alone as shown in figure 5.20 (upper panel). Consequently, these findings closely parallel the outcome of the experiments presented earlier, where autophagy inhibition enhanced sensitivity to radiation but reversed the enhancement of radiation sensitivity by EB 1089. These studies support the premise that autophagy has diametrically opposite functions in the case of radiation alone (cytoprotective) and radiation + EB 1089 (cytostatic/radiosensitizing).

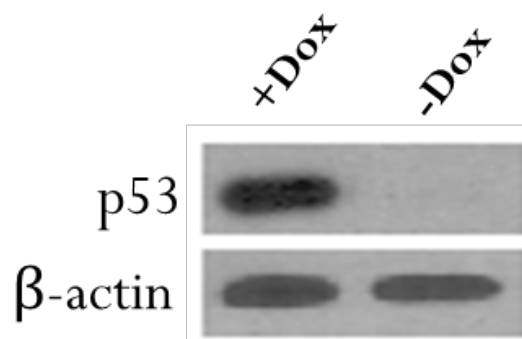


Figure 5.1. Induction of p53 in H1299 cells indicated by western blotting. Western blotting indicating expression of p53 in the presence doxycycline (1ug/ml) of in H1299 cells, which are otherwise p53 null.

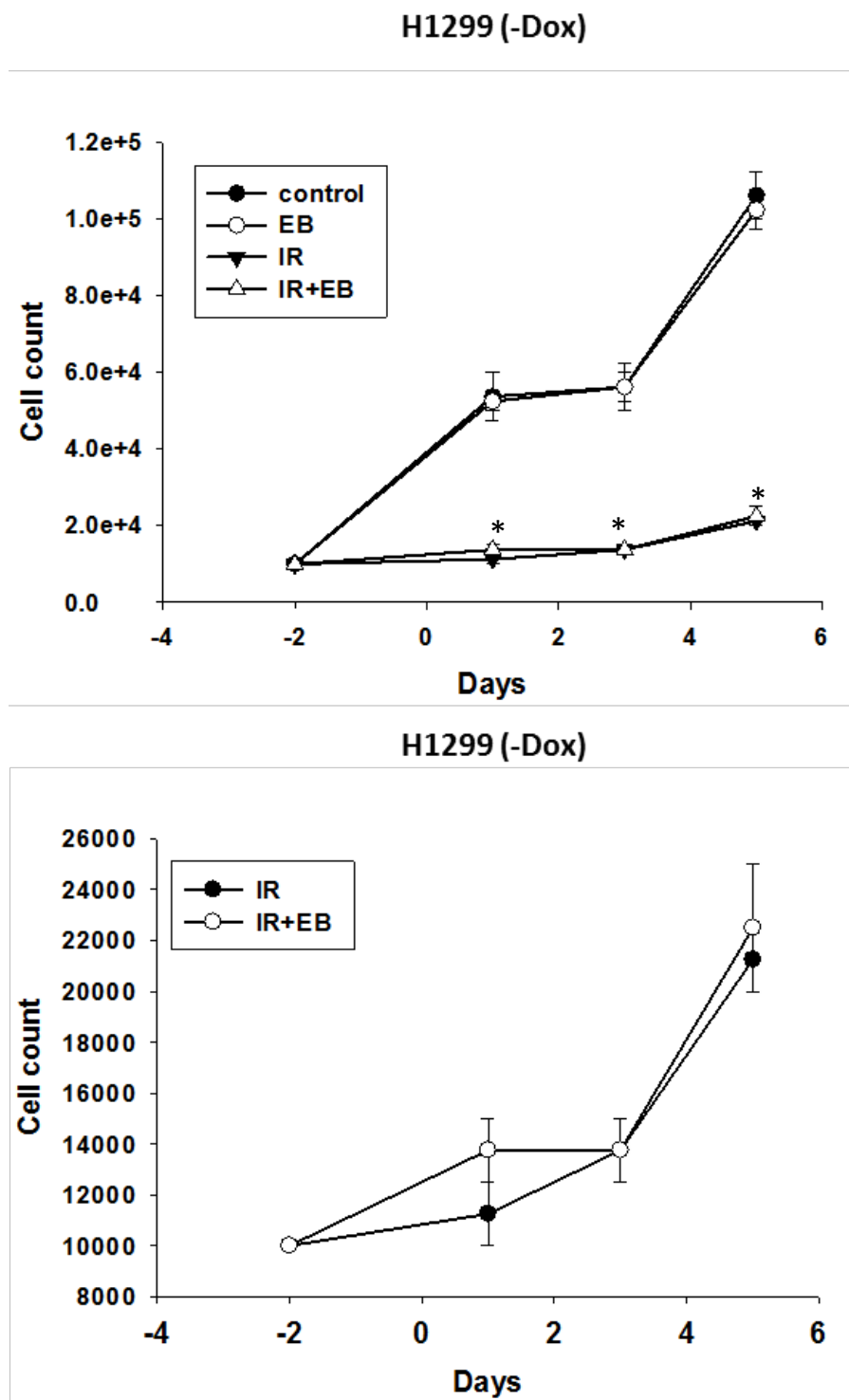


Figure 5.2. Lack of radiosensitization by EB 1089 in p53 null (-Dox) H1299 cells. Cell viability studies indicating lack of sensitization to IR+EB 1089 treatment in p53 null H1299 (-Dox) (n=3, mean \pm SE; *p<0.05 compared to control). No radio-sensitization was observed with the combination treatment of IR + EB 1089.

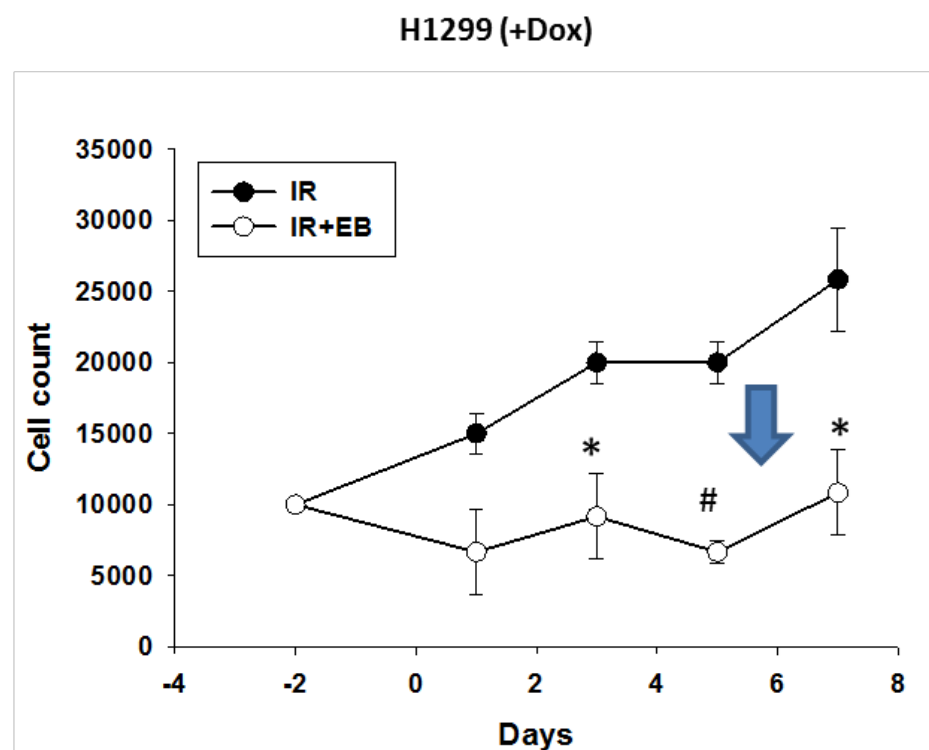
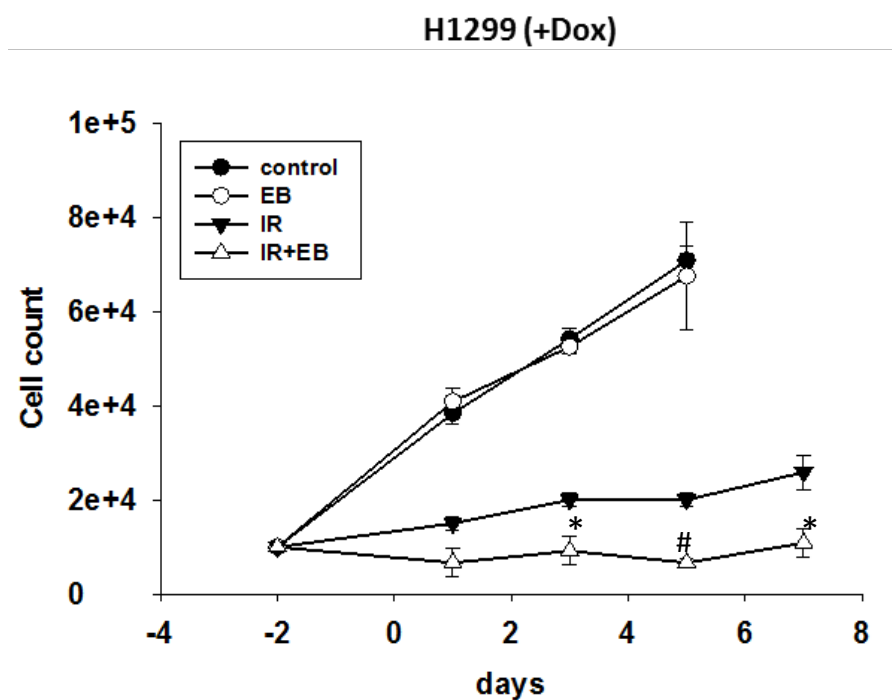
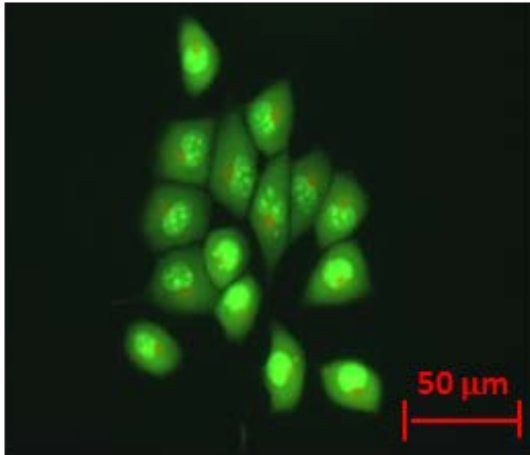


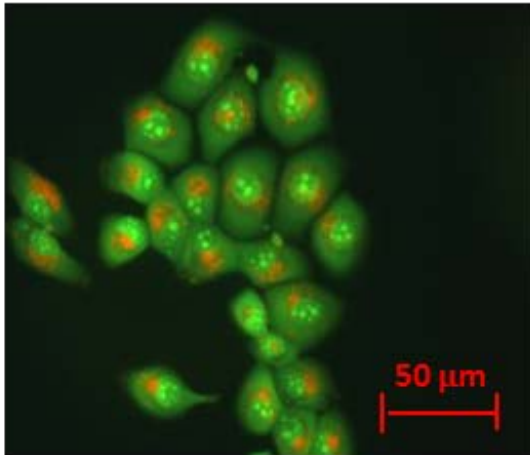
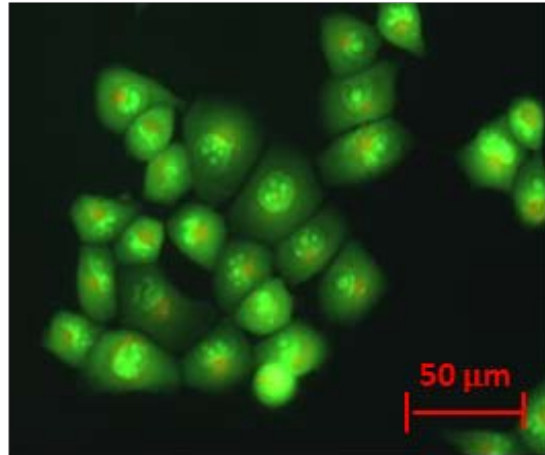
Figure 5.3. Radiosensitization by EB 1089 in p53 induced (+Dox) H1299 cells. Cell viability studies indicating sensitization to IR+EB 1089 treatment in p53 induced H1299 (+Dox) (n=3, mean \pm SE *p<0.05, #p<0.001 as compared to IR alone). Significant amounts of radio-sensitization was observed for the combination treatment of IR+ EB 1089 as compared to IR alone

H1299 (-Dox)

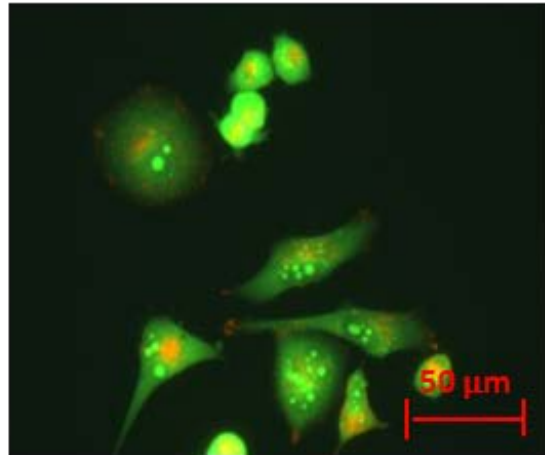
Control



EB 1089



IR

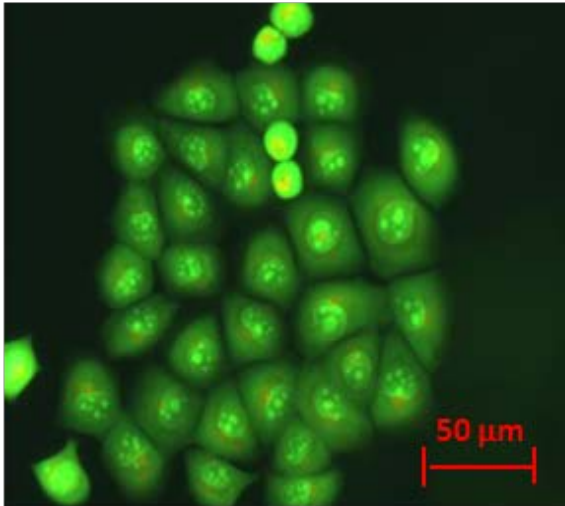


IR+EB 1089

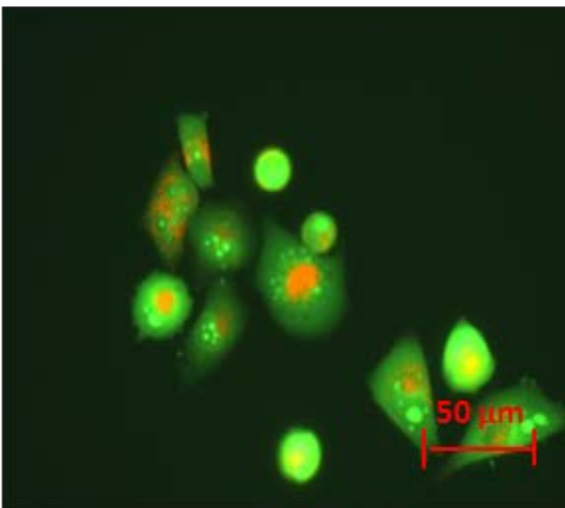
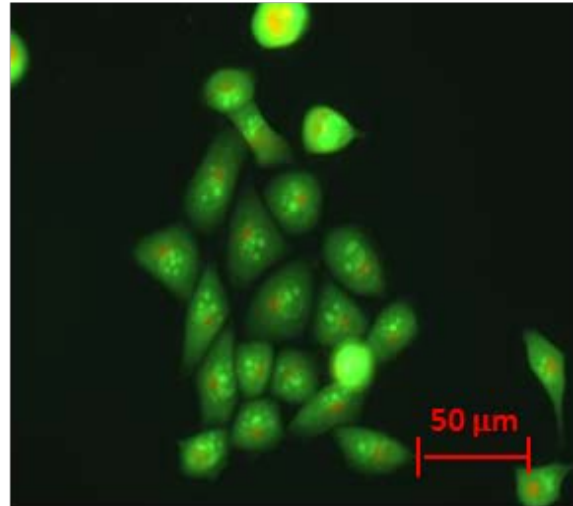
Figure 5.4. Autophagy induction in p53 null (-Dox) H1299 cells. Acridine orange staining indicating acidic vesicle formation in response to IR and IR+EB1089 treatment in H1299 (-Dox) cells on day 3. (all images were taken at the same magnification. Moderate amounts of autophagy is induced by the IR and IR+EB 1089 treatments.

H1299 (+Dox)

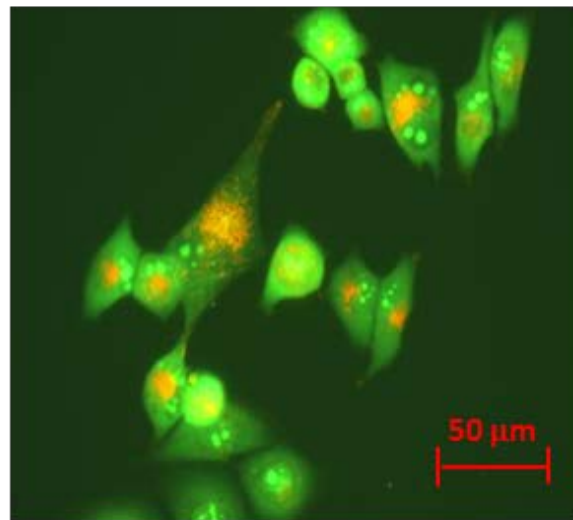
Control



EB 1089



IR



IR+EB 1089

Figure 5.5. Autophagy induction in p53 induced (+Dox) H1299 cells. Acridine orange staining indicating acidic vesicle formation in response to IR and IR+EB1089 treatment in H1299 (+Dox) cells on day 3. (all images were taken at the same magnification).

H1299 (-Dox)

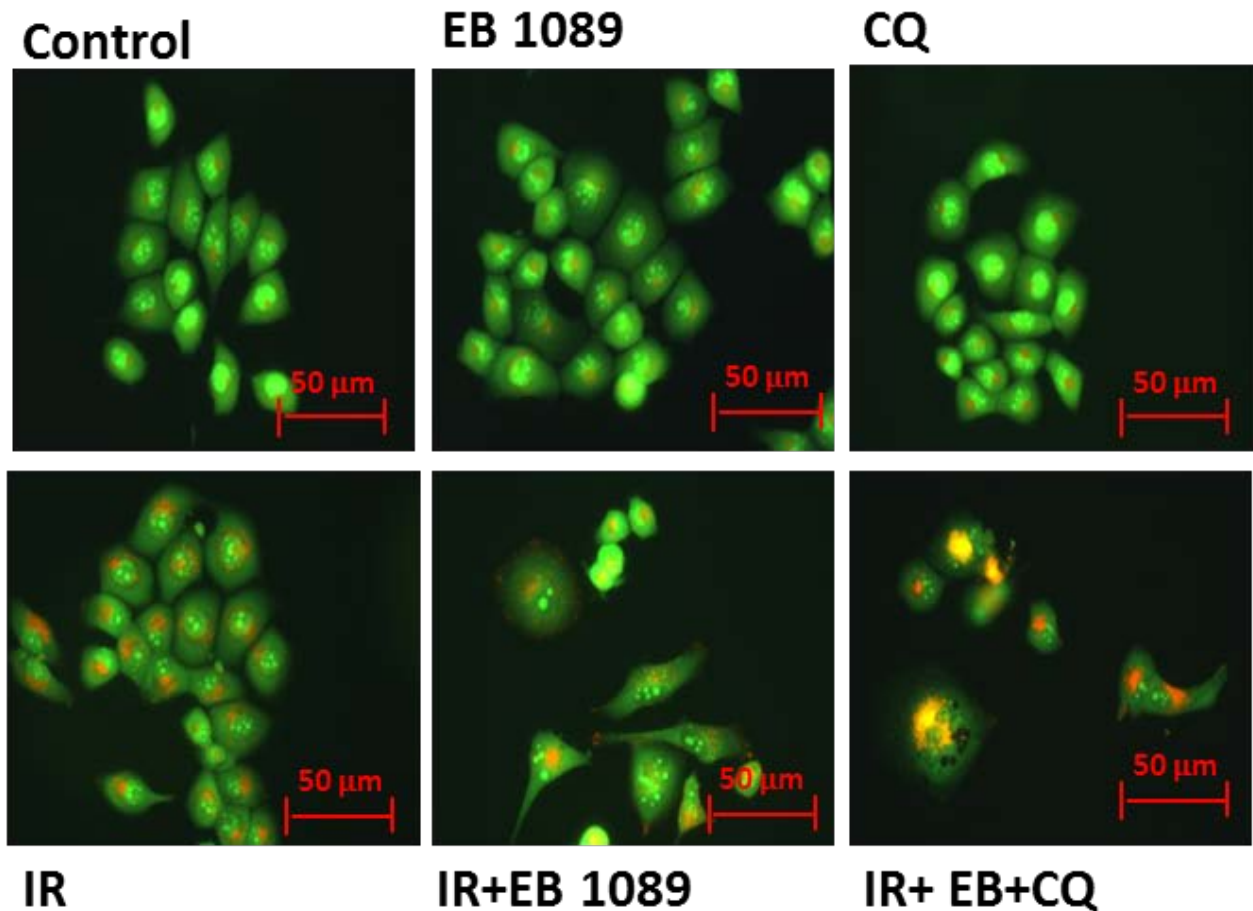


Figure 5.6. Inhibition of autophagy in p53 null (-Dox) H1299 cells by chloroquine. Inhibition of autophagy indicated by increased acidic vesicle formation with use of late stage autophagy inhibitor, chloroquine, in H1299 (-Dox) cells by day 3 (all images were taken at the same magnification). Being a weak base, chloroquine alters the pH of the acidic vesicles and can further lead to the color change in acidic vesicles from orange to yellow.

H1299 (+Dox)

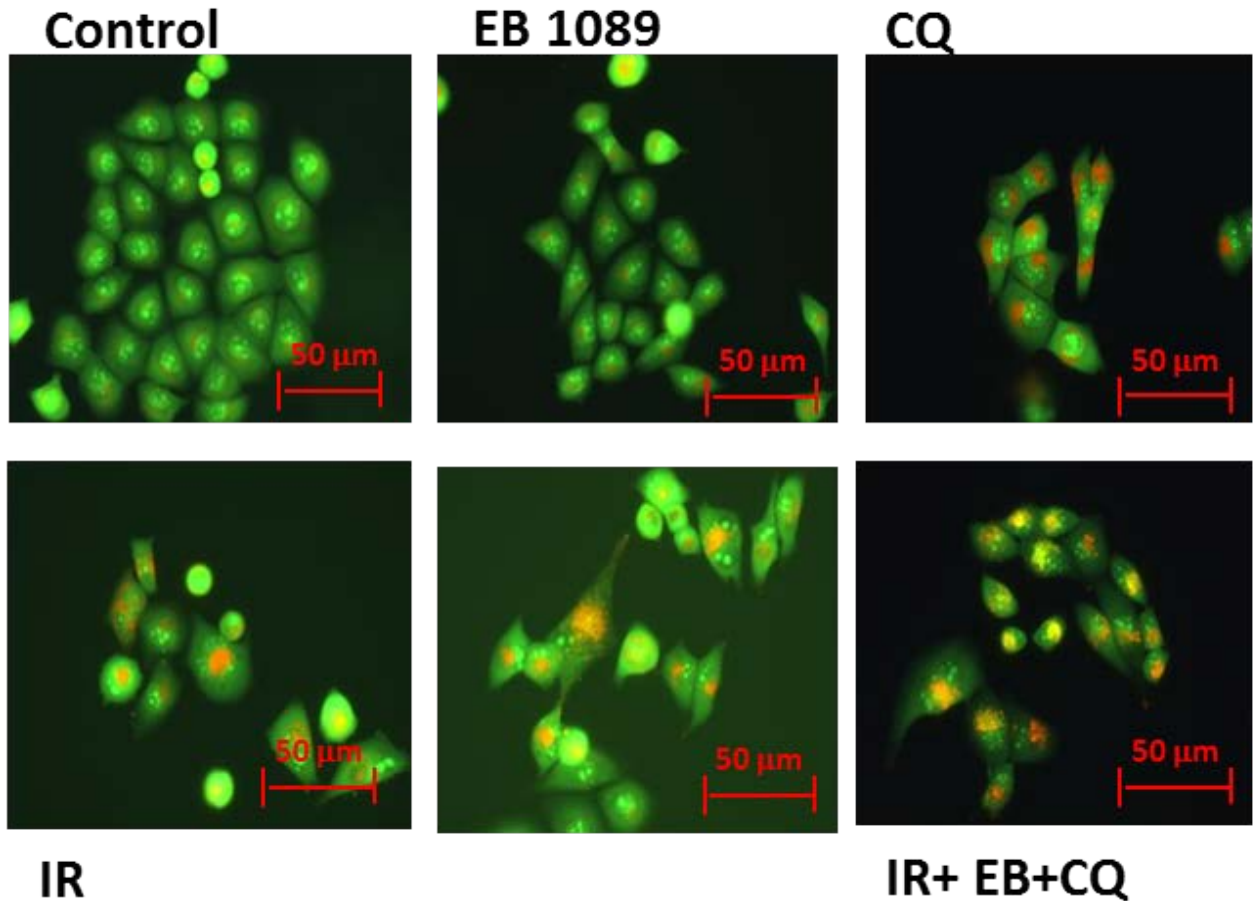
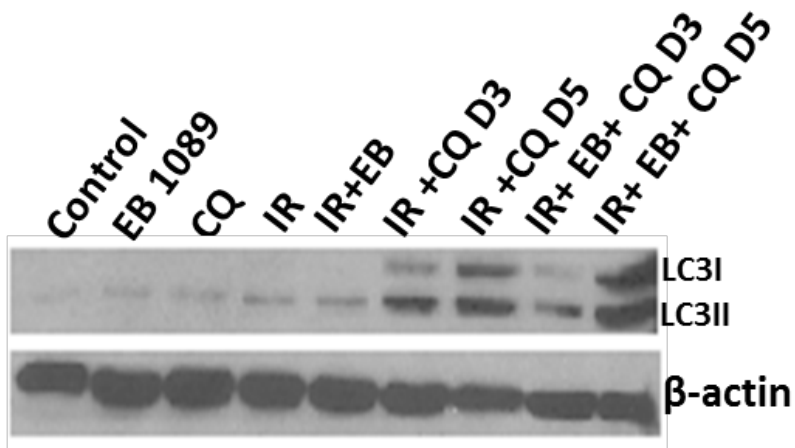


Figure 5.7. Inhibition of autophagy in p53 inducible (+Dox) H1299 cells by chloroquine. Inhibition of autophagy indicated by increased acidic vesicle formation with use of late stage autophagy inhibitor, chloroquine, in H1299 (-Dox) cells) on day 3. (all images were taken at the same magnification). Being a weak base, chloroquine alters the pH of the acidic vesicles and can further lead to a color change in vesicles from orange to yellow.

H1299 (-Dox)



H1299 +Dox)

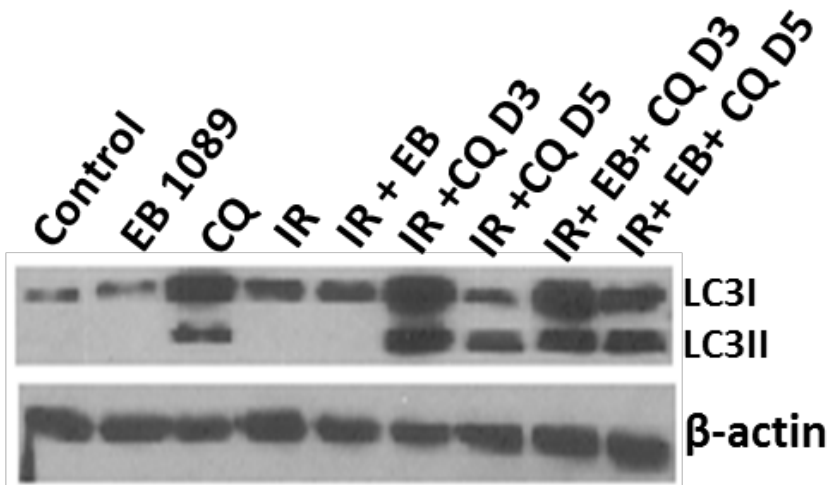


Figure 5.8. Inhibition of autophagic flux in H1299 cells by western blotting. Interference with autophagic flux as indicated by accumulation of LC3II by Western blotting in H1299 (-Dox) and (+Dox) cells on day 3. Accumulation of LC3II indicates accumulation of autophagosomes.

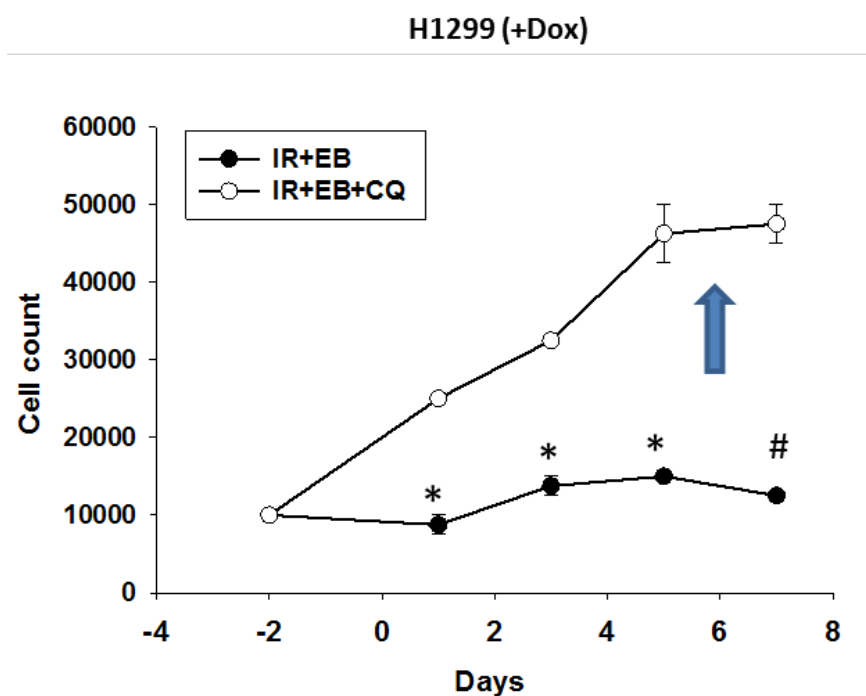
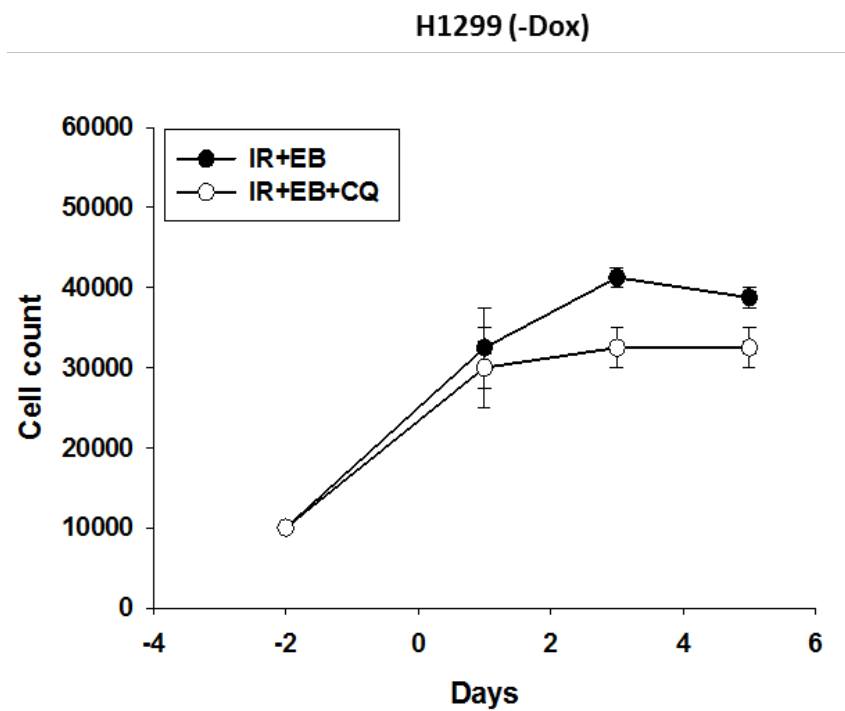


Figure 5.9. Blocking autophagy reverses the sensitization in p53 inducible (+Dox) H1299 cells. Cell viability studies indicating no change in sensitivity upon autophagy inhibition in p53 null H1299 (-Dox) cells (upper panel). Cell viability studies indicating reversal in sensitivity upon autophagy inhibition by pharmacological inhibitor, chloroquine, in p53 induced H1299 (+Dox) cells (lower panel) (n=3, mean \pm SE; *p<0.05; #p<0.001). These studies were carried out for a time period of 7 days.

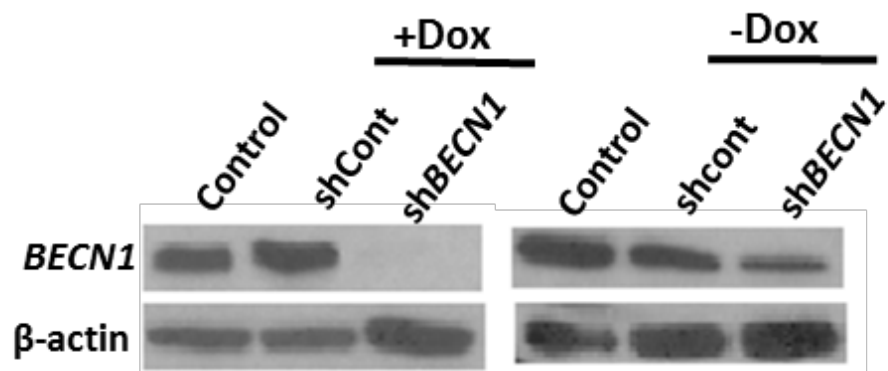
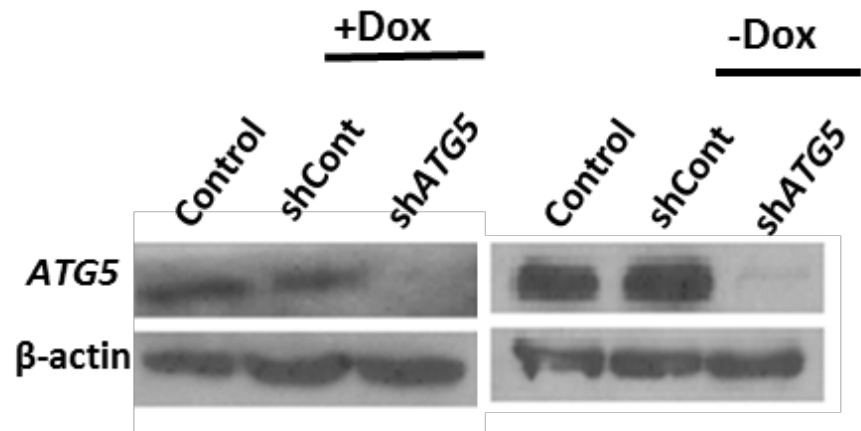


Figure 5.10. Genetic knockdown of autophagy genes H1299 cells. Western blotting indicating knock down of autophagy genes *ATG5* (upper panel) and *BECN1* (lower panel) in both H1299 (-Dox) and H1299 (+Dox) cells.

H1299 (-Dox)

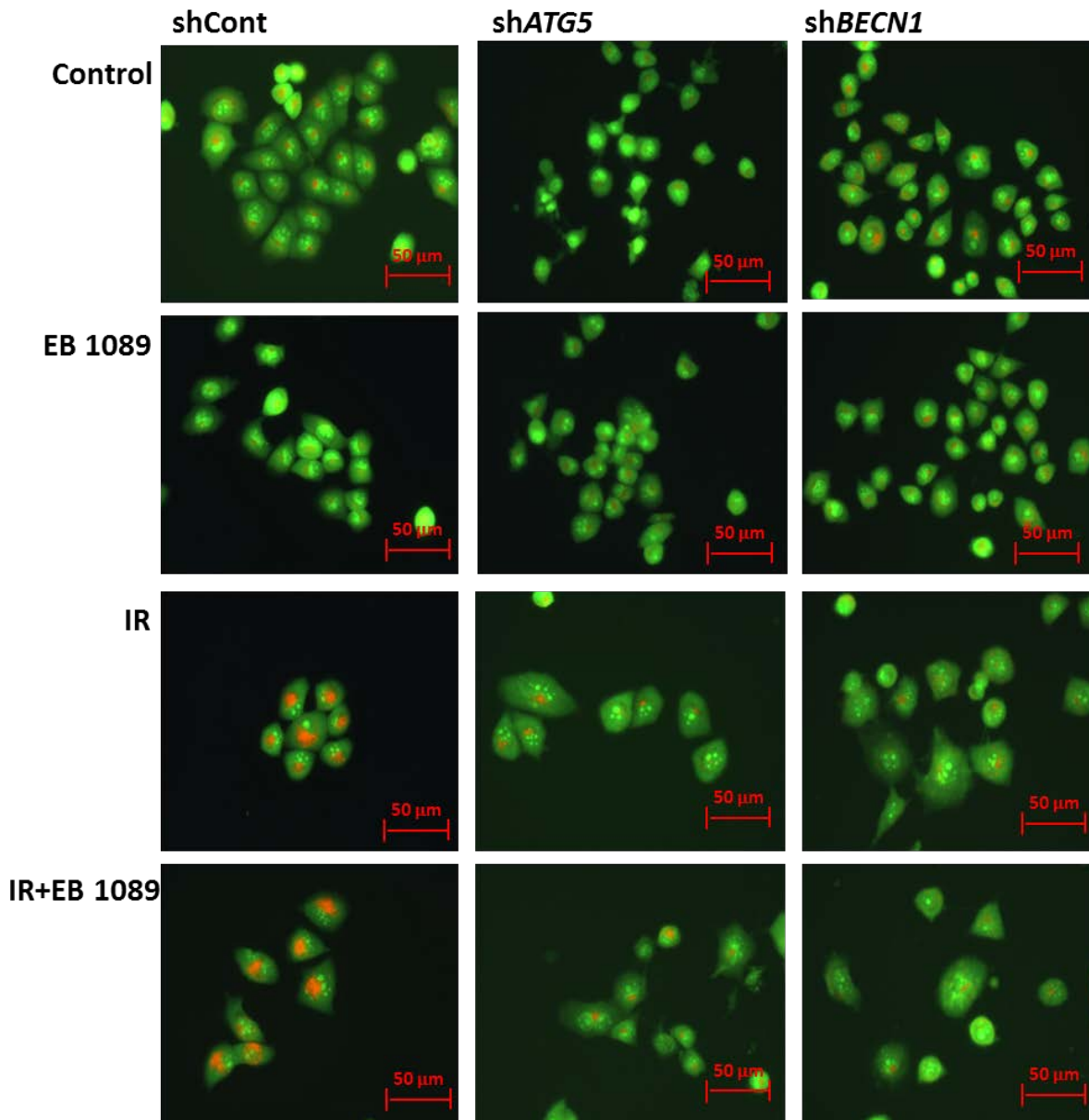


Figure 5.11. Autophagy inhibition in p53 null (-Dox) H1299 cells by knockdown of autophagy related genes. Acridine orange staining indicating inhibition of autophagy after *ATG5* and *BECN1* knockdown in H1299 (-Dox) cells on day 3. Orange color indicating acidic vesicles is significantly reduced when autophagy genes are knocked down in response to IR and combination treatment of IR+ EB 1089.

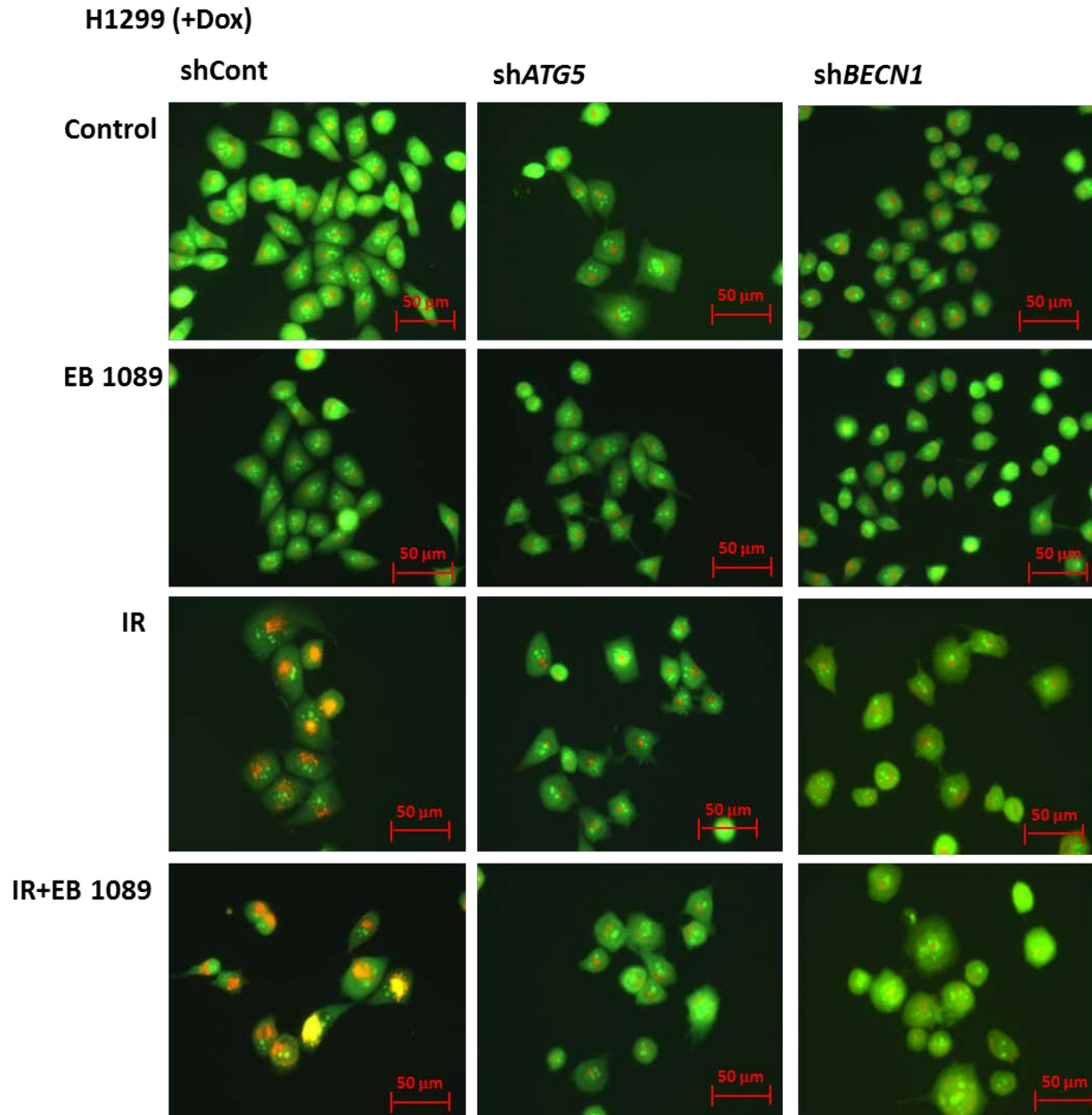


Figure 5.12. Autophagy inhibition in p53 inducible (+Dox) H1299 cells by knockdown of autophagy related genes. Acridine orange staining indicating inhibition of autophagy after *ATG5* and *BECN1* knockdown in H1299 (-Dox) cells. Orange color indicating acidic vesicles is significantly reduced when autophagy genes are knocked down in response to IR and combination treatment of IR+ EB 1089.

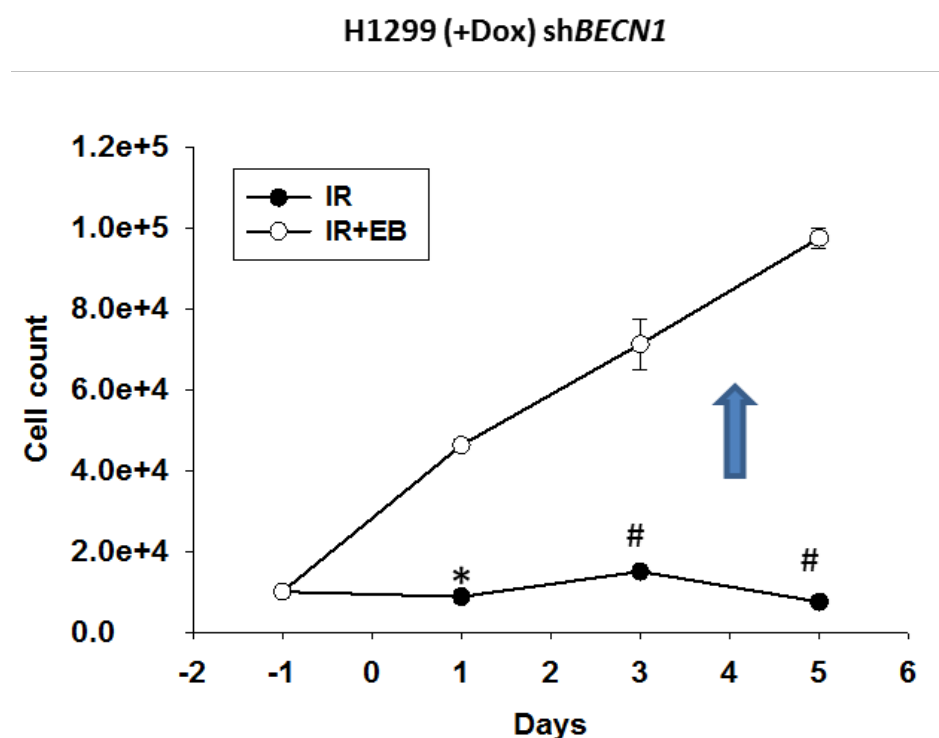
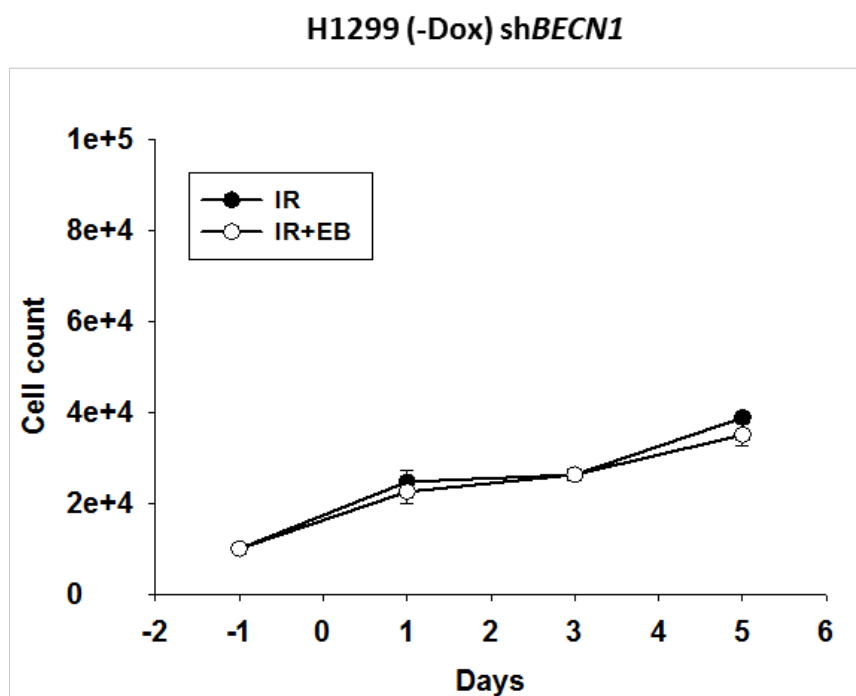


Figure 5.13. Blocking autophagy by knocking down *BECN1* reverses the sensitization in p53 inducible (+Dox) H1299 cells. Cell viability studies indicating no change in sensitivity upon autophagy inhibition in p53 null H1299 (-Dox) cells (upper panel). Cell viability studies indicating reversal in sensitivity upon autophagy inhibition by knocking down *BECN1* in p53 induced H1299 (+Dox) cells (upper panel) (n=3, mean \pm SE; *p<0.05; #p<0.001). These studies were carried out for a time period of 5 days.

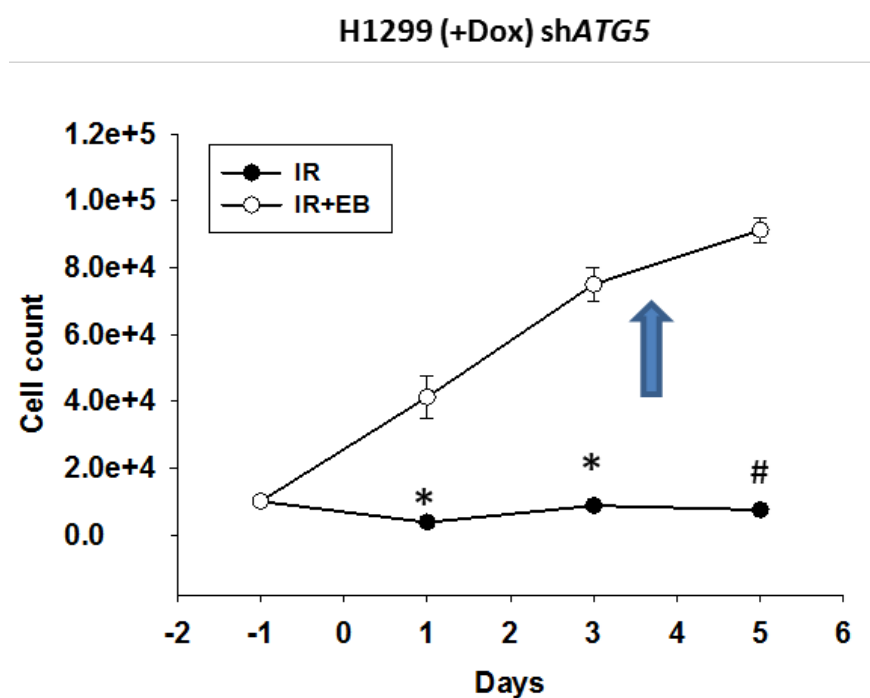
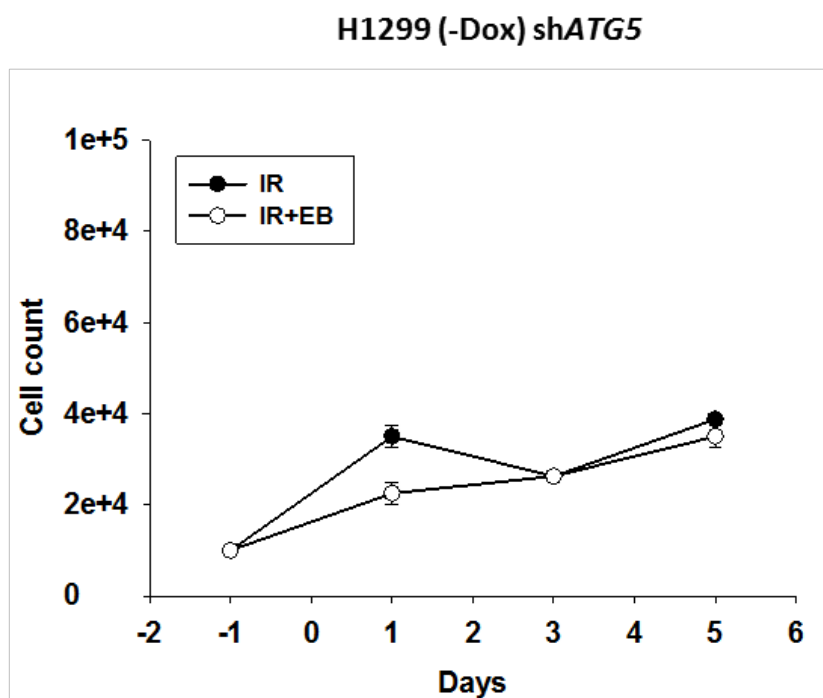


Figure 5.14. Blocking autophagy by knocking down *ATG5* reverses the sensitization in p53 inducible (+Dox) H1299 cells. Cell viability studies indicating no change in sensitivity upon autophagy inhibition in p53 null H1299 (-Dox) cells (upper panel). Cell viability studies indicating reversal in sensitivity upon autophagy inhibition by knocking down Atg5 in p53 induced H1299 (+Dox) cells (lower panel) (n=3, mean \pm SE; *p<0.05; #p<0.001). These studies were carried out for a time period of 5 days.

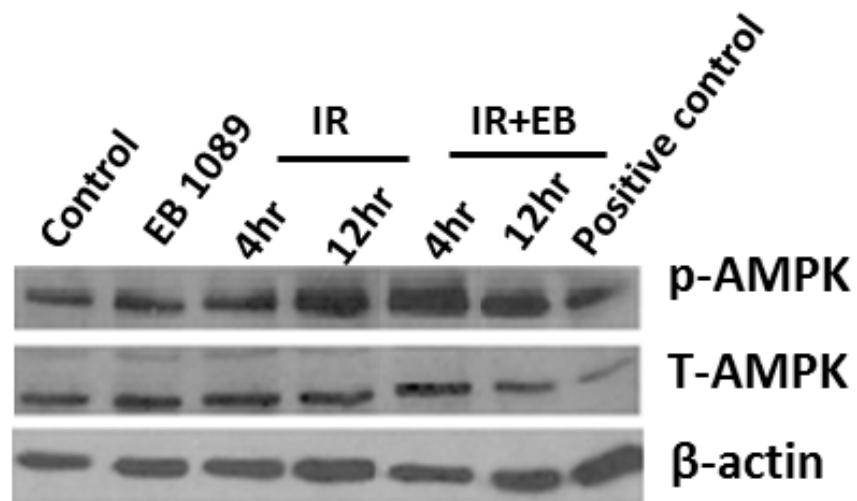


Figure 5.15. Induction in phosphorylation of AMPK in response to IR and IR+EB 1089 treatments. Western blotting indicating changes in phosphorylation levels of AMPK in response to various treatments at early time points, 4 and 12 hours. Phospho-AMPK is significantly up regulated as early as 4 hours and stays so till 12 hours.

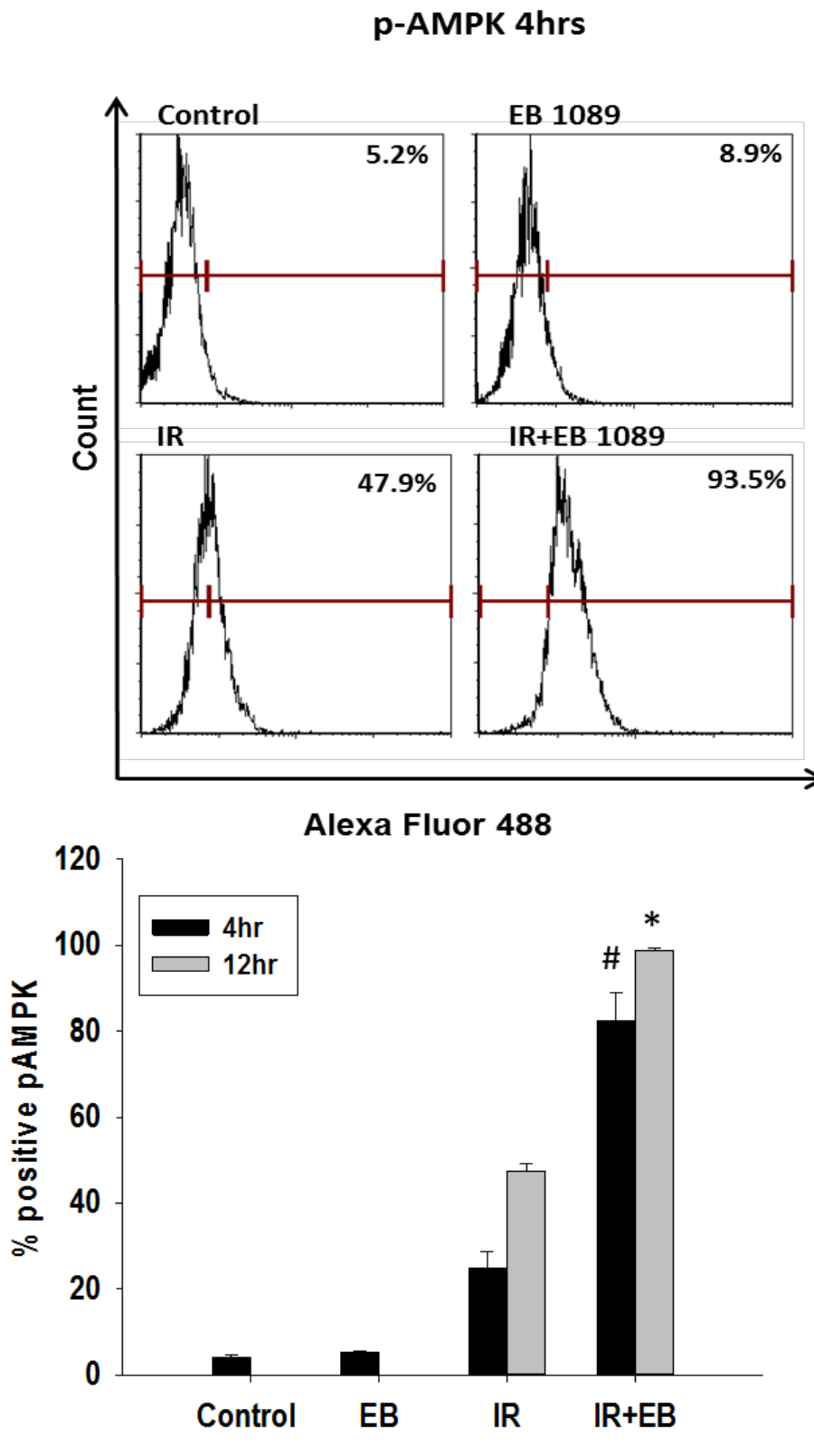


Figure 5.16. Assessment of changes in AMPK phosphorylation by flow cytometry. Assessment of phosphorylation of AMPK (Thr172) by flow cytometry at 4 and 12 hours post-radiation (n=3, mean \pm SE, *p<0.05, #p<0.001). Significant increase in phospho-AMPK levels with the combination treatment of IR+ EB 1089 as compared to EB treatment alone at 4 and 12 hours is observed.

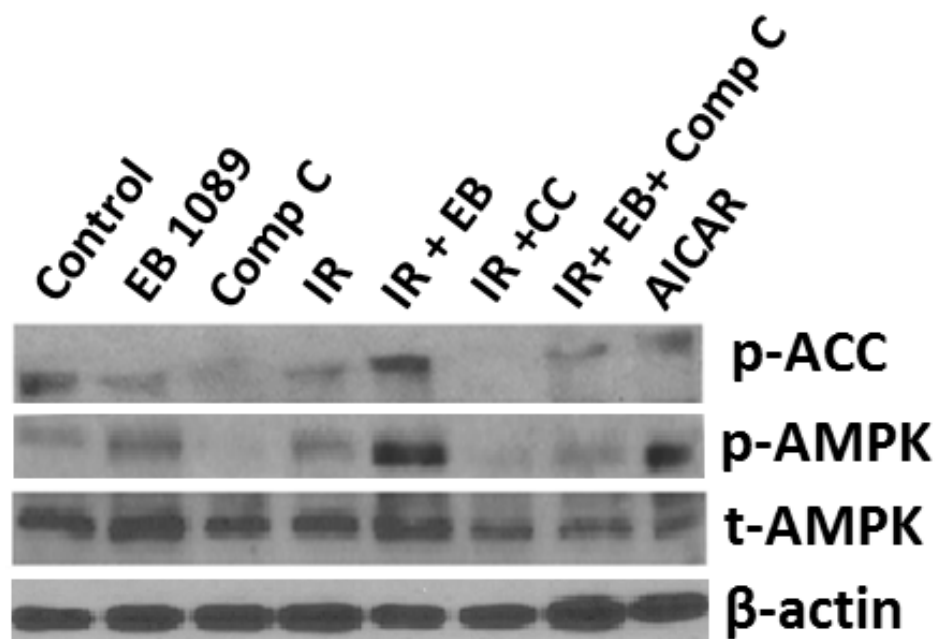


Figure 5.17. Inhibition of phosphorylation of AMPK and ACC by compound C. Western blotting indicating inhibition of phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase (ACC) by compound C (inhibitor of AMPK). Samples were collected 12 hours after different treatments were given.

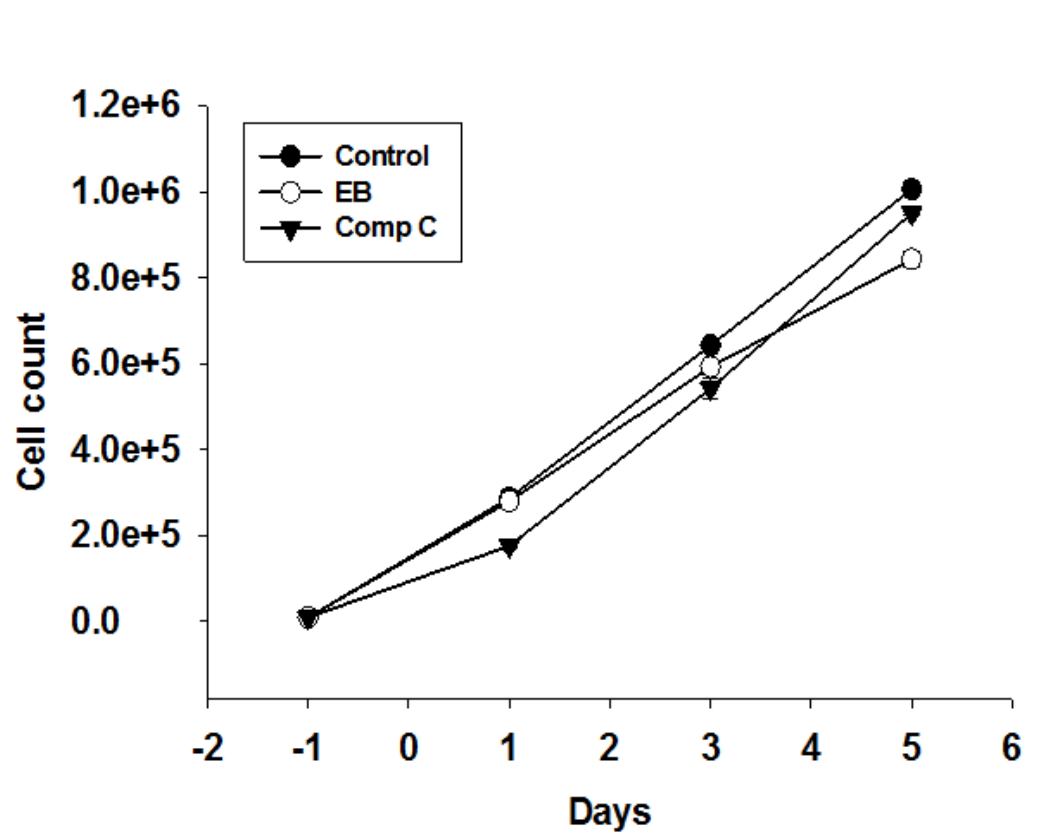


Figure 5.18. Minimal effect of compound C on cell viability. Cell viability studies indicating minimal growth inhibitory effects of compound C used at 5 μ M concentration in H460 cells (n=3, mean \pm SE). Cells were treated with compound C for 48 hours and cell viability was assessed over a time period of five days.

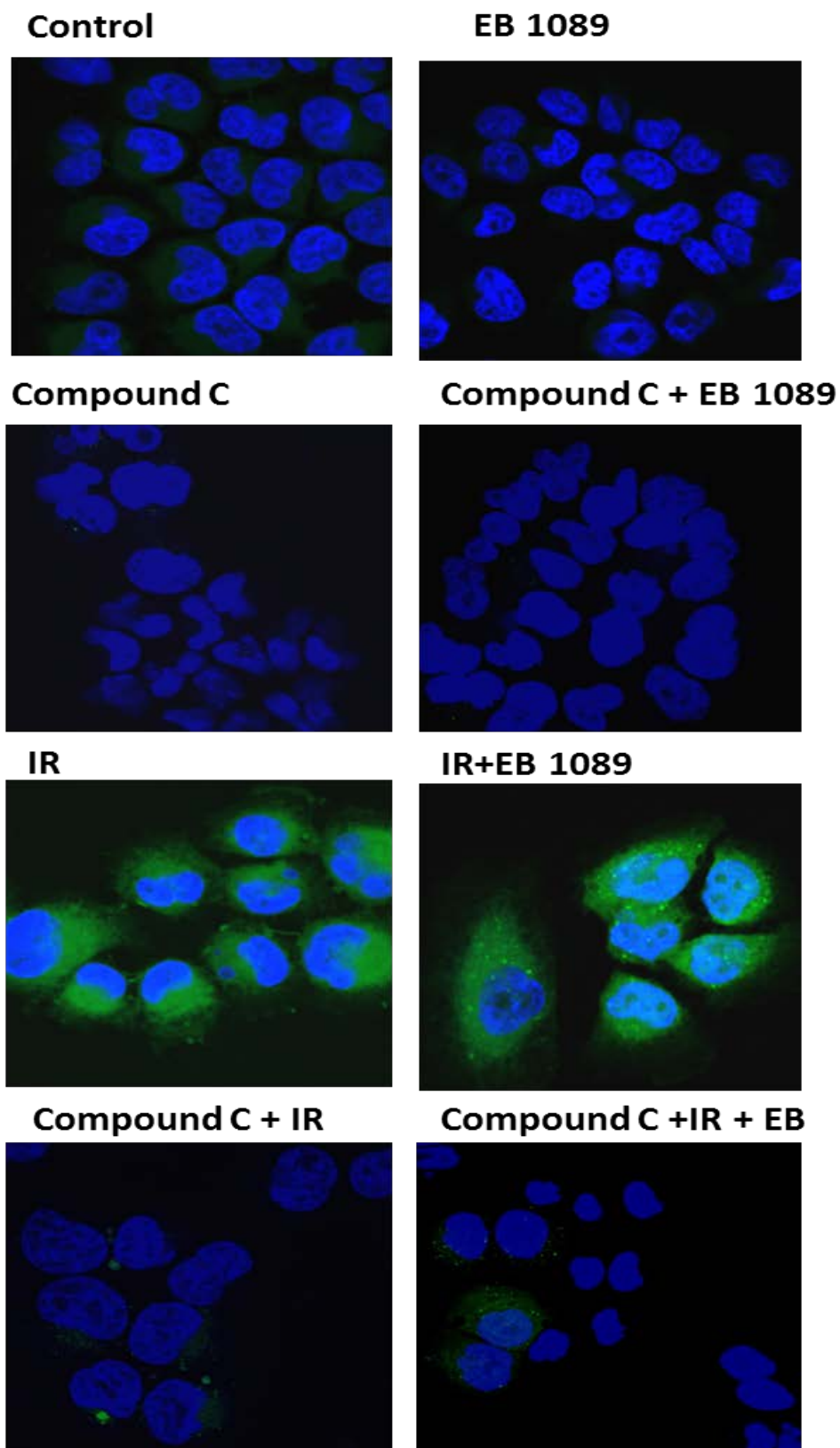


Figure 5.19. Assessment of GFP-LC3 redistribution after inhibition of AMPK using compound C. GFP-LC3 transfected H460 cells were exposed to 100 nM EB 1089 and IR (6Gy) treatment with and without compound C (5 μ M). 12 hours after treatment cells were fixed and counterstained with DAPI and visualized using confocal microscope.

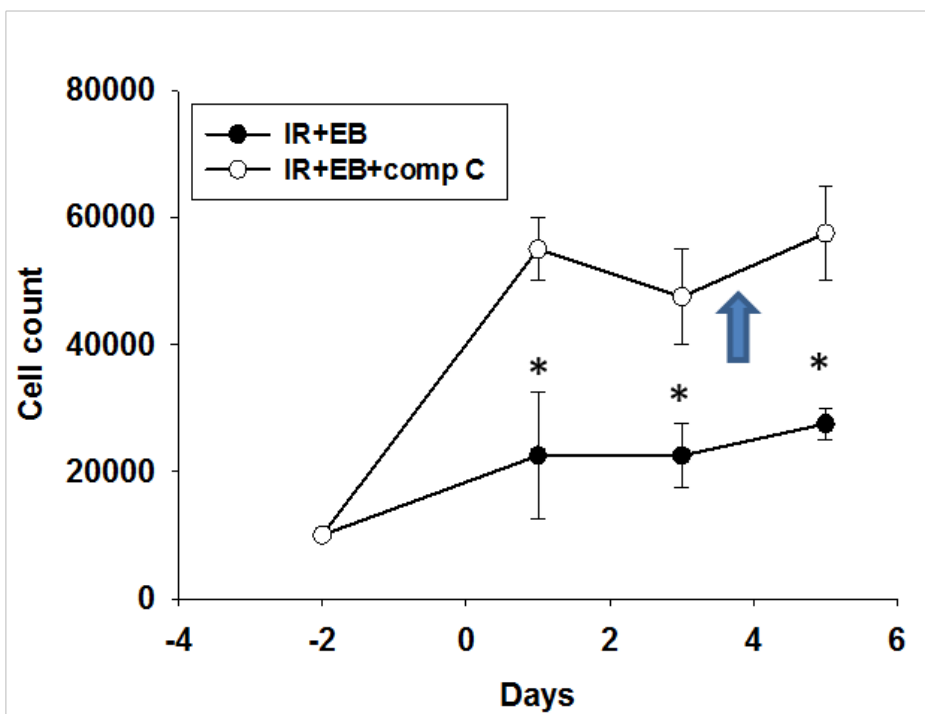
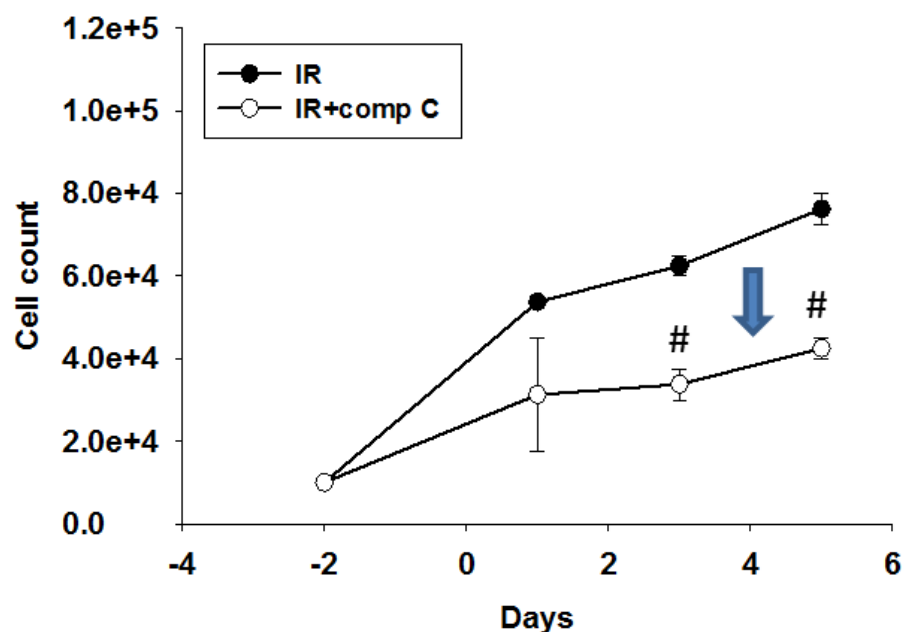


Figure 5.20. Effects of pharmacological inhibition of AMPK on cell viability. Cell viability studies conducted to assess the effects of Compound C (5 μ M) on the temporal response to radiation (IR, 6 Gy) alone or EB 1089 + radiation in h460 cells (n=3, mean \pm SE, *p<0.05, #p<0.001). Inhibiting AMPK in response to IR treatment, cell were sensitized as indicated by a reduction in cell count. However, inhibiting AMPK in response to IR+EB 1089, resulted in reversal of sensitization.

5.3. Summary.

Studies conducted in p53 mutant and p53 null cell lines, H358 and H1299, indicate the importance of wild-type p53 in order to sensitize tumor cells to radiation using vitamin D and its analogues. Lack of sensitization in p53 mutant and null cell lines and regain of sensitivity in H1299 cells when p53 was expressed led us to our conclusion. We employed both pharmacological inhibition and genetic inhibition of autophagy to study the interlink between autophagy, p53 and radiosensitization in NSCLC cells.

Studies assessing levels of AMPK indicated its involvement in both IR and IR + EB 1089 induced autophagy although the increase was significantly higher for the combination treatment. Levels of phosphorylated ULK-1 also appeared to be higher for the combination treatment as compared to IR treatment alone. Treatment with compound C, a pharmacological inhibitor of AMPK, resulted in decreased autophagy and reversed radiosensitization obtained with the combination treatment. Furthermore, it increased sensitization when used for radiation alone, complementary to our earlier studies done with pharmacological and genetic inhibition of autophagy. These studies suggest that AMPK is involved and plays an important role in both functions of autophagy but does not appear to be the differentiating factor between the two forms, cytoprotective and cytostatic. However, since pharmacological inhibitors can have off target effects, studies using a genetic approach to inhibit AMPK will be done to validate these findings.

Chapter 6

Discussion

The treatment of lung cancer continues to represent a clinical challenge. While current treatments do often prolong patient life, these therapies almost invariably become primarily palliative (Saintingy P, *et al.* 2012; Wintner LM, *et al.* 2013). Therefore, there is a compelling need to identify and develop strategies to make the existing treatments more effective.

Radiation is the gold standard lung cancer treatment (Cai S, *et al.* 2014; Bayman N, *et al.* 2013; Hamamoto Y, *et al.* 2012); however, disease recurrence has always been an issue. One of the main goals of our laboratory has been to develop strategies to attenuate the recovery of tumor cells after radiation treatment. In addition, the doses of radiation that can be administered are limited by toxicity to normal adjoining tissue (Van Kaick G, *et al.* 2008). Consequently, increasing the selectivity of radiation therapy would be of great benefit to lung cancer patients.

A number of studies in the past have been reported on the effectiveness of combining 1,25-D₃ or vitamin D analogues with conventional chemotherapies such as Tamoxifen or Adriamycin against a variety of tumors in preclinical studies (Hershberger PA, *et al*, 2001; Wang Q, *et al*, 2000; Light BW, *et al*. 1997). Vitamin D alone has been shown to have anti-proliferative effects in various experimental models of cancer (Krishnan AV, *et al*. 2012; Ramnath N, *et al*. 2011). However, in our hands, 1,25-D₃ or EB 1089 alone, at 100nM, showed no antiproliferative or cytotoxic effects against the NSCLC cell lines (H460, A549, H1299 and H358) used in the current work despite high expression of the vitamin D receptor; this highlights that fact that the effects of the combination treatments are not merely additive but reflect a mechanistic interaction that is likely to be related to the induction of autophagy (since vitamin D by itself has minimal inhibitory effects on the growth of cells).

It is important to emphasize that our studies differ from other in which vitamin D alone had effects on cell growth or viability in that we do not utilize a continuous treatment; vitamin D and EB 1089 are removed from cells after 48 hours. Furthermore, the dose used in our work (100 nM) is not in the high physiological range and can be achieved pharmacologically in patients (Ilahi M, *et al*. 2008; Hillyer RL, *et al*. 2012)

Similar to the findings with breast tumor cells, the presence of the VDR is critical since studies conducted with p53 wild-type H838 NSCLC cells, which lack the vitamin D receptor, showed lack of radio-sensitization with the combination treatment.

Drug selectivity and specificity are critical elements in efforts to avoid damage to normal tissue. Non-specific drug effects can result in off-target actions causing severe and

undesired side effects. We showed that vitamin D does not increase radiation sensitivity in normal bronchial epithelial cells in spite of their expression of the vitamin D receptor (Jeong Y, *et al.* 2012) or in H9C2 cardiomyocytes which have wild-type p53 (Yang YF, *et al.* 2011), the latter being relevant in cases where the heart might fall within the radiation field during the course of treatment.

Previous work from our laboratory has shown that vitamin D has the capacity to enhance radio-sensitivity in breast tumor cells (Bristol ML, *et al.* 2012; Wilson EN, *et al.* 2011; Demasters GA, *et al.* 2004; Sundaram S, *et al.* 2003), mainly through promotion of autophagy (Bristol ML, *et al.* 2012; Wilson EN, *et al.* 2011; Demasters GA, *et al.* 2006). We sought to extend these findings to non-small cell lung tumor cells, which are relatively resistant to radiation treatment. Our studies provide support for the potential use of vitamin D in the development of adjuvant therapy for non-small cell lung cancer treatment.

6.1 Sensitization to radiation:

One of the most common responses to stress generated by radiation is DNA damage, where radiation results in either single strand or double strand breaks (Ward JF, *et al.* 1998). Therefore, we first used flow cytometry to assess DNA damage in response to radiation treatment by quantifying the amount of phosphorylated γ -H2AX. We observed an increase in DNA damage as early as 1 hour after irradiation of the cells, which then was quickly repaired. However, there was no difference either in the extent of initial DNA damage or the rate of repair for radiation alone and vitamin D + radiation.

While the combination treatment of radiation + 1,25D₃ or EB 1089 resulted in cell death in breast tumor cells (Bristol ML, *et al.* 2012; Wilson EN, *et al.* 2011), in the current studies we observe primarily extended growth inhibition and suppression of proliferative recovery. One of the reasons for this difference could be because lung tumors are known to be relatively more radio-resistant as compared to breast tumors

The combination treatment of radiation + 1,25D₃ or EB 1089 did not increase either apoptosis or necrosis, similar to what we have reported previously for breast tumor cells (Bristol ML, *et al.* 2012; Wilson EN, *et al.* 2011). In contrast to the studies in breast tumor cells, there was no evidence of cell death (i.e. a decline in viable cell number over time). Nevertheless, we observed unequivocal evidence for radio-sensitization in clonogenic survival assays, which is consistent with a reduction in the capacity for cellular self-renewal. The observed impact on self-renewal capacity and proliferative recovery could prove to be relevant to the problem of disease recurrence in lung cancer patients.

Since our cell viability studies indicate a primary response of growth inhibition and studies suggest induction of senescence in response to radiation in breast tumor cells (Jones KR, *et al.* 2005; Wilson EN, *et al.* 2011), we used beta-galactosidase staining to assess levels of senescence in A549 and H460 cell lines. We also employed flow cytometry to quantify the extent of senescence. Although senescence induction was evident with both IR and IR+ 1,25-D₃/EB 1089 treatments, we did not observe a significant increase in senescence when radiation was combined with 1,25-D₃ or EB 1089.

Since we have previously shown that sensitization in breast tumor can occur by induction of autophagy, induction of autophagy was shown in the non-small cell lung cancer models using multiple complementary assays including acridine orange staining/flow cytometry, GFP-LC3 puncta formation by confocal microscopy and western blotting to estimate levels of autophagic flux related proteins, LC3II and p62.

6.2 Radiation treatment alone induces cytoprotective function of autophagy.

A number of studies in the literature support the premise that autophagy produced in response to radiation is cytoprotective in nature (Ko A, *et al.* 2014; Apel A, *et al.* 2008; Cheng G, *et al.* 2013). We have confirmed that radiation-induced autophagy is cytoprotective in our cell models as the cells are sensitized to radiation when autophagy is blocked using either pharmacological inhibitors such as chloroquine and bafilomycin or genetic tools to knockdown autophagy genes, *ATG5* and *BECN1*.

We performed cell viability studies to study the growth profile and performed annexinV/PI staining to estimate alterations in levels of apoptosis when autophagy was blocked. Induction of apoptosis when autophagy is inhibited has been shown previously in the literature (Cheng G, *et al.* 2013). Studies by Apel A, *et al.* (2008) demonstrated the cytoprotective nature of autophagy in A549 NSCLC cells. Interestingly, this effect was more pronounced in cells with wild-type p53. We observed a similar trend in our studies where we obtain cytoprotective autophagy in response to radiation in wild-type p53 cell lines, A549 and H460.

In a study published by Kroemer's research group (Kroemer G, *et al.* 2008), it is argued that autophagic cell death does not exist and that autophagy simply constitutes a death

effector mechanism when upstream of other cell death pathways such as apoptosis or necrosis. This work supports a pro-survival/protective role of autophagy produced in response to radiation alone (Kroemer G, *et al.* 2008).

Although our studies of radiation-induced autophagy are consistent with a cytoprotective function, we observe a novel form/function of autophagy when vitamin D is employed in combination with the radiation treatment. We have termed this new form “cytostatic” since it is associated with enhanced growth arrest upon IR + 1,25-D3/EB 1089 treatment as shown in cell viability studies. Of particular importance, inhibition of this form/function of autophagy leads to a reversal in sensitization. Furthermore, we do not observe any induction in cell death modes such as apoptosis or necrosis with the combination treatment.

6.3 Radio-sensitization by vitamin D caused by a change in the function of autophagy.

While our initial experiments in A549 cells suggested that sensitization might be related to an *increase* in the extent of autophagy, since we see significant differences in autophagy levels with radiation alone and the combination treatment of IR and 1,25-D3, subsequent experiments in H460 cells tended to rule out this possibility; that is, there was only a minimal and largely non-significant increase in the extent of autophagy with the combination treatment

Inhibition of autophagy induced in response to the combination treatment of IR and EB 1089 using both pharmacological and genetic tools led to a reversal in sensitization in H460 cells. These findings in particular support the relatively novel premise that the

cytoprotective function of autophagy that is evident with radiation alone is converted to a cytostatic function (pronounced growth inhibition, suppression of proliferative recovery and reduced clonogenic survival) with the combination treatment of IR and EB 1089. This conclusion is supported by the observations that inhibition of autophagy sensitizes the cells to radiation alone but protects the cells from the combination treatment. Our argument for the conversion of one form of autophagy to the other is that sensitization to radiation through autophagy inhibition appears to have been entirely eliminated from the experimental system; otherwise, we might have expected sensitization to radiation by inhibition of the protective autophagy induced by radiation alone to have masked the *reversal* of sensitization to EB 1089 or 1,25-D₃ when cytostatic autophagy was inhibited. If radiation induced cytoprotective autophagy was still prevalent in response to the combination treatment, then inhibition of the autophagy induced in response to the combination treatment would not result in reversal of the sensitization to radiation.

This idea of a switch in autophagic function has also been reported previously in the literature. The combination treatment of vitamin D and IR has been shown to cause autophagy to switch from a cytoprotective to a cytotoxic function in breast tumor cells (Wilson EN, *et al.* 2011; Bristol ML, *et al.* 2012). Certain chemotherapies such as topotecan and camptothecin have been reported to cause similar changes in the function of autophagy from cytoprotective to cytotoxic in colon carcinoma cells and osteosarcoma cells (Li DD, *et al.* 2012; Hollomon MG, *et al.* 2013). Although the mechanisms underlying these changes have not yet been identified, these studies indicate that autophagy can demonstrate opposing functions in a particular

malignancy/cancer cell type and therefore, increase the challenge of exploiting the radiation or chemotherapy induced autophagy for therapeutic gain.

6.4 Efforts to identify signaling pathways associated with autophagy:

Our previous work established that p53 is required for sensitization to radiation by vitamin D in breast tumor cells (Bristol ML, *et al.* 2012; Wilson EN, *et al.* 2011) and this appears to be the case for NSCLC cells as well, since H358 cells with mutant p53 did not demonstrate any radio-sensitization by EB 1089. Furthermore, sensitization to radiation by EB 1089 could not be shown in the p53 null, VDR expressing H1299 cell line unless p53 expression was induced under the influence of doxycycline. A linkage between p53 status and the biological impact of vitamin D has been reported previously (Stambolsky P, *et al.* 2010). These studies demonstrated that mutant p53 could convert the anti-proliferation effects of vitamin D into pro-proliferation effects, which could potentially explain the lack of sensitization in the absence wild-type p53. Therefore, there seems to be a requirement for wild-type functional p53 for vitamin D to be able to act as a radio-sensitizer and produce its anti-proliferative effects.

Another study reported by Reichrath J *et al.*(2014) indicated that there is evidence of crosstalk between vitamin D signaling and p53. Both p53 and VDR act as transcriptional regulators, which are present at the core of the common network involved in regulation of proliferation, survival and homeostasis. VDR has been shown to have a p53 binding sites (Maruyama R, *et al.* 2006). The literature suggests that wild-type p53 can lead to activation and induction of VDR. VDR and p53 engage in physical interaction (indicated by co-immunoprecipitation), which has been shown to be significantly enhanced in the

presence of vitamin D (Maruyama R, *et al.* 2006; Stambolsky P, *et al.* 2010). Regulation of both p53 and VDR can lead to alteration of a number of p53 target genes (Stambolsky P, *et al.* 2010). Taken together, these studies suggest that the interaction of functional p53 and VDR might be playing a role in 1,25 D3/EB 1089 induced radiosensitization by altering their downstream targets.

There are multiple signaling pathways that can mediate the link between cellular damage and autophagy, and p53 appears to play a dual in this crosstalk (Sui X, *et al.* 2011). p53 is involved in regulating autophagy through transcriptional activation of the AMP-activated protein kinase (AMPK), which is a potent inducer of autophagy (Cheng G, *et al.* 2013; Alexander A, *et al.* 2011; Feng Z, *et al.* 2005). The requirement for functional p53 is consistent with the apparent involvement of AMPK in autophagy as p53 has been shown to act upstream of AMPK (Cheng G, *et al.* 2013; Lorin S, *et al.* 2013). Additionally, Feng *et al.* (2005) showed activation of AMPK in a p53 dependent manner and further indicated that p53 can bind to the promoter of β subunit of AMPK as it has a consensus p53 binding site (Feng Z, *et al.* 2007). . However, this activation appears to be stress specific as it occurs in response to UV radiation but not when Adriamycin treatment is given (Feng Z, *et al.* 2007). In our studies, p53 appears to be essential for activation of AMPK but we do not yet have evidence that p53 directly promotes phosphorylation of AMPK since transactivation would be expected to induce an increase in levels of AMPK To investigate p53's involvement further, we would plan to transfect p53 null H1299 cells with p53 mutated specifically in its transactivation domain to determine whether p53 acting as a transcriptional activator (possibly at other target sites) is responsible for AMPK activation (phosphorylation).

AMPK can also be activated by CaMKK β (Ca²⁺/calmodulin-dependent kinase kinase β) (Woods A, et al. 2005; Hawley SA, et al. 2005), upon recognition of increased Ca²⁺ levels, which has been linked to vitamin D (Hoyer-Hansen *et al.* 2007). Furthermore, studies have implicated radiation in the activation of AMPK (Sanli T, *et al.* 2010). In efforts to define the pathways involved in sensitization effects of vitamin D, we designed studies to identify and establish AMPK mediated autophagy, as both vitamin D and radiation are involved in AMPK regulation. Additionally, studies in MCF-7 breast tumor cells have shown proof of up-regulation of phospho-AMPK and phospho-ULK-1 in response to the combination treatment of radiation and 1,25-D₃ as compared to radiation treatment alone (manuscript in press).

Experiments were conducted to study changes in phosphorylation levels of AMPK in response to IR or the combination treatment of IR+EB 1089 in H460 cells. Assessment of AMPK activity revealed up-regulation of phosphorylated AMPK (Thr172 phosphorylation site) as early as 4 hours after exposure to both IR and IR+EB 1089 treatments but the increase was much more significant for the combination treatment. These findings implicate the AMPK pathway in the induction of cytostatic function of autophagy in response to the combination treatment of radiation and EB 1089.

To further establish/study AMPK's role in autophagy induced by radiation and radiation + vitamin D/EB 1089, we studied the effects of the pharmacological inhibitor of AMPK, compound C (Zhou G, *et al.* 2001) on the growth kinetics of cells. Western blotting confirmed compound C's ability to inhibit phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase (ACC). Inhibition of autophagy was confirmed by attenuation of GFP-LC3 redistribution when compound C was used in combination with

IR and EB 1089. Studies monitoring cell viability suggested that inhibition of AMPK by compound C also sensitized the cells to radiation while protecting the cells from the combination treatment of radiation + EB 1089, complementary to the studies assessing the impact of more direct autophagy inhibition. Our studies indicated that blocking autophagy by inhibiting AMPK phosphorylation leads to a reversal in sensitization in response to the combination treatment of IR + EB 1089 but an increase in sensitization in response to IR, further strengthening the idea/concept of two different functions of autophagy.

The observation that blocking AMPK phosphorylation using compound C was able to alter the functions of autophagy for both IR and the combination treatment of IR+EB 1089 suggests that AMPK might not be the distinguishing factor between the two different functions of autophagy. However, AMPK definitely appears to play a role in vitamin D mediated sensitization and provides valuable insight into the signaling pathways being activated leading to autophagy by IR and the combination treatment of IR + EB 1089.

One of the most interesting and novel findings of our studies is the switch in function of autophagy from cytoprotective in response to radiation to cytostatic in response to IR+EB 1089. One of the simple explanations for this switch could be that the initial response to stress in a system is protective in nature where the cell is trying to maintain homeostasis and activates all the pro survival mechanisms but when the stress becomes intolerable (as might be the case with the combination treatment) and homeostasis cannot be maintained, the function changes from being protective to static/toxic where either arresting the growth of cells or destroying them is the only

alternative. One other explanation could be that when cells are exposed to radiation, the initial tendency is towards self-protection, resulting in activation of signaling pathways that lead to induction of cytoprotective autophagy. However, the presence of vitamin D/EB 1089 is somehow masking the activation/up-regulation of some of these pathways and therefore causing the cells to undergo growth arrest to gain more time to repair the damage.

6.5 Autophagy and senescence relationship?

Studies in the literature have suggested a link between autophagy and senescence (Narita M, *et al.* 2009; Gewirtz DA, 2009; Young AR, *et al.* 2010). It would be interesting to examine the relationship between autophagy and senescence in the NSCLC model since the two processes occur at the same time. Furthermore, senescence has been implicated as a mechanism of autophagy-mediated dormancy (Lu Z, *et al.* 2008; Gewirtz DA, 2009). Selected autophagy genes such as ULK1 and ULK3 have been shown to be upregulated during senescence (Young AR, *et al.* 2009). Employing tools to inhibit autophagy and assess the effects on senescence is something we would like to do in the future.

Previous studies from our laboratory also indicate that IR can induce senescence in breast tumor cells in a p53 dependent manner (Jones KR, *et al.* 2005). Using the H1299 p53 null/inducible model could further shed some insight on the link/relationship between autophagy and senescence in the absence and presence of functional p53.

6.6 Clinical Trials

A number of studies both in-vitro and in-vivo have demonstrated vitamin D's ability to promote anti-tumor effects when used in combination with chemotherapy (McGuire MF, *et al.* 2001; Colston KW, *et al.* 1993; Wilson EN, *et al.* 2011). However there are a number of severe limitations when it comes to translating these findings to the clinic. Even though results from preclinical and clinical studies have indicated that high doses of vitamin D can be administered safely and feasibly either alone or in combination with other agents to enhance its anti-tumor activity (Woloszynska-Read A, *et al.* 2011), the optimal dose or concentration that can be used safely in the clinic is yet to be determined. Possibly as a consequence of the fact that optimal doses of vitamin D were not used, a number of clinical trials did not succeed as expected (Flaig TW, *et al.* 2006; Beer TM, *et al.* 2003; Muindi JR, *et al.* 2009). Furthermore, in our studies we have shown that both VDR and p53 are essential for vitamin D and its analog EB 1089 to mediate its growth inhibitory effects when used in combination with radiation. These studies indicate the need for stratification of patients based on their VDR and p53 status to improve the outcomes of any clinical trials involving vitamin D or its analogs.

A recent clinical trial failed to demonstrate the advantage of combining calcitriol (vitamin D) with cisplatin in NSCLC, likely to be due to lack of stratification with regard to vitamin D receptor and p53 status in a relatively small cohort of near-terminal patients (Ramnath N, *et al.* 2013). As chemoradiation utilizing cisplatin and/or vinca alkaloids is the standard of care for NSCLC (Eberhardt W, *et al.* 2005), our future studies will include evaluation of the combination of 1,25-D₃/EB 1089 with cisplatin/radiation and

vinca alkaloids/radiation in cell culture, which will ultimately be extended to tumor bearing animals.

6.7 Future studies

Clinical relevance of the use of vitamin D has been gaining attention over the last decade. Studies have suggested that 1,25-D₃ is able to interfere with lung metastasis (Ingraham BA, *et al.* 2008). Expression of VDR and high circulating levels of the vitamin D precursor, 25(OH)D₃ have been associated with prolonged survival in NSCLC cancer patients (Anderson MG, *et al.* 2006). Studies have further linked vitamin D deficiency to increased incidence and high mortality rates in breast, prostate and lung cancer patients (Abbas S, *et al.* 2008; Bertone-Johnson ER, *et al.* 2005; Lowe *et al.* 2005; Porojnicu *et al.* 2008). However, there is insufficient information as to the mechanisms underlying vitamin D's ability to have anti-cancer effects when combined with either radiation or chemotherapy.

There is an extensive amount of evidence in the literature implicating the ER stress pathway as being involved in autophagy induction (Digaleh H, *et al.* 2013). Studies have suggested that both 1,25-D₃ and IR can induce ER stress (Hoyer-Hansen *et al.* 2007; Kim, K.W., *et al.* 2010). Our future studies will be designed to explore the ER stress pathway to determine if it is involved in the radio-sensitization effects produced by vitamin D and furthermore if this signaling pathway consists of factors that can differentiate between the two functions of autophagy.

It is possible that vitamin D is able to sensitize tumor cells to radiation by activating multiple pathways to induce autophagy. Some studies conducted in breast tumor and

leukemia have suggested that 1,25-D₃ has the ability to down regulate bcl-2 which is an anti-apoptotic gene and binds to an autophagy inducing gene, Beclin-1 (Van Leeuwen and Pols, 2005). Down-regulation of bcl-2 could lead to more free Beclin-1, which could explain why 1,25-D₃ radio-sensitizes tumor cells by inducing autophagy.

Another possible explanation for different functions of autophagy could be related to differences in selective cargo that is degraded in the autophagosome. Recently a study by Chen S, *et al.* (2014) indicated that inhibiting p62, which is a known signaling adapter and is involved in carrying ubiquitinated proteins and cellular components for degradation into autophagosomes, impairs the process of cargo loading into autophagosomes. Inefficient autophagy further leads to accumulation of the BH3-only protein NBK/Bik, which causes apoptosis. In order to investigate whether the content of autophagosome is different (for radiation alone and EB 1089 + radiation) we propose to isolate the autophagosome and analyze its contents by using mass spectrometry. It is possible that when autophagy is protective in nature, the autophagosome content is not harmful to the cells but when autophagy is cytostatic (or cytotoxic) in nature then the content released is harmful and the cells undergo growth arrest to repair the damage.

We will also be performing parallel studies using another approach of proteomics (mass spectrometry) to complement this effort. Specifically our goal would be to identify phosphoproteins that distinguish between cytoprotective and cytostatic functions of autophagy in order to ultimately use these as biomarkers in the clinic to predict which tumors would be susceptible to autophagy inhibition as a strategy for sensitization to radiation.

A recent review indicated that the subunits of AMPK, $\alpha 1$ and $\alpha 2$, could have different localization patterns, with the $\alpha 2$ subunit being localized in both nucleus and cytoplasm and $\alpha 1$ being restricted to the cytoplasm (non-nuclear fraction) (Kazgan *et al.* 2010; Alexander A, *et al.* 2011). Although previous studies conducted in our laboratory in breast tumor cells showed an increase in both nuclear and cytoplasmic localization of AMPK in response to the combination treatment of IR and vitamin D (data not published), there is a possibility that $\alpha 1$ and $\alpha 2$ are being differentially regulated by the combination treatment, which could be playing a role in changing the function of autophagy. p27, a cyclin dependent kinase inhibitor, is a direct downstream target of AMPK and induces growth arrest by inhibiting cell cycle progression when activated in the nucleus (Alexander A, *et al.* 2011). It could be possible that the cytostatic function of autophagy involves up-regulation of AMPK $\alpha 2$, causing cells to undergo growth arrest in response to the combination treatment. We have adenoviruses that express dominant negative $\alpha 1$ and $\alpha 2$ isoforms and inhibition of each isoform will be employed to test this hypothesis.

Another kinase that has been shown to regulate autophagy is ULK-1, which can be directly activated by AMPK. A study by Kim *et al.* (2011) demonstrated a molecular mechanism for regulation of ULK-1 (Jung *et al* 2010; Kim *et al.* 2011). Under starvation, AMPK promotes phosphorylation of ULK-1 at Ser317 and Ser777, which leads to up-regulation of autophagy (Jung *et al* 2010; Kim *et al.* 2011). Under nutrient sufficiency, high mTOR activity leads to down-regulation of autophagy by phosphorylating ULK-1 Ser757, which disrupts the interaction between ULK-1 and AMPK (Jung *et al* 2010; Kim *et al.* 2011). We would assess levels of both ULK-1 and mTOR to extend and identify

differences in the signaling pathways involved in the two different functions of autophagy.

We also intend to expand these studies to tumor bearing animal models where its pharmacokinetics (absorption, distribution, metabolism, and excretion) will play an important role in its ability to radio-sensitize tumors. Consequently, we intend to perform initial studies to carefully determine the dose range to be employed in animals. We propose to utilize one cell line where vitamin D confers sensitization to radiation (H1299/p53 inducible) and one where vitamin D is ineffective (H1299/p53 null cells) and their isogenic counterparts where autophagy has been genetically inhibited by knocking down autophagy related genes such as atg5 and Beclin-1. The two tumor cell lines (autophagy deficient and autophagy proficient) will be grown as xenografts in opposing flanks, and irradiated providing a direct assessment of sensitization by vitamin D.

6.8 Summary

The current work builds upon and extends our previous findings in breast cancer in demonstrating that 1,25-D₃ and the vitamin D analog EB 1089 enhance sensitivity to radiation in NSCLC cells. Again, as in previous work, sensitization is limited to NSCLC cells that express the vitamin D receptor and functional p53. Furthermore, there is no apparent sensitization in normal lung epithelial cells or cardiomyocytes, consistent with previous work that indicated that sensitization did not occur in non-transformed cells (Polar MK et al. 2003). The current work also is similar to our previous findings in implicating autophagy as the mechanism underlying the radio-sensitization. However, *in contrast* to the promotion of a cytotoxic form of autophagy in breast tumor cells (Bristol ML, et al. 2012; Wilson EN, et al. 2011; Demaster GA, et al. 2006), we present the novel observation that the autophagy induced by radiation in combination with either 1,25-D₃ or EB 1089 has a cytostatic function that nevertheless translates into an enhanced reduction of clonogenic survival. Finally, in these studies, we observe the apparent conversion of the cytoprotective form of autophagy induced by radiation alone to the cytostatic form by radiation in combination with +1,25-D₃ or EB 1089.

References

- Abbas, S., Linseisen, J., Slanger, T., Kropp, S., Mutschelknauss, E.J., Flesch-Janys, D., and Chang-Claude, J. (2008). Serum 25-hydroxyvitamin D and risk of post-menopausal breast cancer-results of a large case control study. *Carcinogenesis* 29,93-99.
- Abida, W. M. and Gu, W. (2008). P53-Dependent and p53-independent activation of autophagy by ARF. *Cancer Res.* 68, 352–357.
- Alexander, A., and Walker, C.L. (2011). The role of LKB1 and AMPK in cellular responses to stress and damage. *FEBS left.* 585(7), 952-7.
- American Cancer Society, T. (2012). Breast Cancer Overview 2012
- American Cancer Society, T. (2014a). What is non-small cell lung cancer? 2014
- American Cancer Society, T. (2014b). How is non-small cell lung cancer treated? 2014
- American Cancer Society, T. (2014c). Radiation therapy- when it is, How it helps.2014
- American Cancer Society, T. (2014d). Advanced cancer overview. 2013. 2014.
- Anderson, M.G., Nakane, M., Ruan, X., Kroeger, P.E., and Wu-Wong, J.R. (2006). Expression of VDR and CYP24A1 mRNA in human tumors. *Cancer Chemother. Pharmacol.* 57(2), 234-40.
- Apel, A., Herr, I., Schwarz, H., Rodemann, H.P., and Mayer, A. (2008). Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. *Cancer res.* 68, 1485-1494.
- Ballinger, S.W., Patterson, C., Yan, C.N., Doan, R., Burow, D.L., Young, C.G., Yakes, F.M., Van Houten, B., Ballinger, C.A., Freeman, B.A., Runge, M.S. (2000). Hydrogen peroxide- and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. *Circ. Res.* 86:960–966.
- Baltayiannis, N., Chandrinou, M., Anagnostopoulos, D., Zaroqoulidis, P., Tsakiridis, K., Mpakas, A., Machairiotis, N., Katsikogiannis, N., Kouqioumtzi, I., Courcoutsakis, N., and Zaroqoulidis, K. (2013). Lung cancer surgery: an up to date. *J. Thorac. Dis.* 425-39.
- Beauséjour, C.M., A. Krtolica, F. Galimi, M. Narita, S.W. Lowe, P. Yaswen, and J. Campisi. (2003). Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J.* 22, 4212–4222.

Beer, T.M., Eilers, K.M., Garzotto, M., Egorin, M.J., Lowe, B.A., and Henner, W.D. (2003). Weekly high-dose calcitriol and docetaxel in metastatic androgen-independent prostate cancer. *Journal of Clinical Oncology* 21(1), 123–128.

Bertone-Johnson, E.R., Chen, W.Y., Holick, M.F., Hollis, B.W., Colditz, G.A., Willett, W.C., and Hankinson, S.E. (2005). Plasma 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D and risk of breast cancer. *Cancer Epidemiol. Biomarkers Prev.* 14, 1991-1997.

Bertrand, R., Solary, E., O'Connor, P., Kohn, K.W., and Pommier, Y. (1994). Induction of a common pathway of apoptosis by staurosporine. *Exp. Cell Res.* 211, 314-321.

Biokoy, G., Lamark, T., Pankiv, S., Overvatn, A., Brech, A., and Johansen, T. (2009). Monitoring autophagic degradation of p62/SQSTM1. *Methods Enzymol.* 452,181-97

Boe, R., Gjertsen, B.T., Vintermyr, O.K., Houge, G., Lanotte, M., and Doskeland, S.O. (1991). The protein phosphatase inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cells. *Exp. Cell. Res.* 195, 237-246.

Bristol, M.L., Di, X., Beckman, M.J., Wilson, E.N., Henderson, S.C., Maiti, A., Fan, Z. and Gewirtz, D.A. (2012). Dual functions of autophagy in the response of breast tumor cells to radiation: cytoprotective autophagy with radiation alone and cytotoxic autophagy in radiosensitization by vitamin D3. *Autophagy.* 8, 739–53.

Bristol, M.L., Emery, S.M., Maycotte, P., Thorburn, A., Chakradeo, S., and Gewirtz, D.A. (2013). Autophagy inhibition for chemosensitization and radiosensitization in cancer: do the preclinical data support this therapeutic strategy. *J. Pharmacol. Exp. Ther.* 344(3), 544-52.

Brücher, B.L., Lyman, G., van Hillegersberg, R., Pollock, R.E., Lordick, F., Yang, H.K., Ushijima, T., Yeoh, K.G., Skricka, T., Polkowski, W., Wallner, G., Verwaal, V., Garofalo, A., D'Ugo, D., Roviello, F., Steinau, H.U., Wallace, T.J., Daumer, M., Maihle, N., Reid, T.J. 3rd, Ducreux, M., Kitagawa, Y., Knuth, A., Zilberstein, B., Steele, S.R., Jamall, I.S. (2014). Imagine a world without cancer. *BMC Cancer.* 14,186.

Cai, S., Shi, A., Yu, R., and Zhu, G. (2014). Feasibility of omitting clinical target volume for limited-disease small cell lung cancer treated with chemotherapy and intensity-modulated radiotherapy. *Radiat. Oncol.* 9,17.

Chaachouay, H., Ohneseit, P., Toulany, M., Kehlbach, R., Multhoff, G., Rodemann, H.P. (2011). Autophagy contributes to resistance of tumor cells to ionizing radiation. *Radiother. Oncol.* 99:287-292.

Chan, E.Y., Longatti, A., McKnight, N.C., and Tooze, S.A. (2009). Kinase-inactivated ULK proteins inhibit autophagy via their conserved C-terminal domains using an Atg13-independent mechanism. *Mol. Cell. Biol.* 29,157–71.

Chang, Y.Y., and Neufeld, T.P. (2009). An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. *Mol. Biol. Cell.* 20, 2004–14.

Chaudhry, M., Sundaram, S., Gennings, C., Carter, H., and Gewirtz, D.A. (2001). The vitamin D3 analog, ILX-23-7553, enhances the response to adriamycin and irradiation in MCF-7 breast tumor cells. *Cancer Chemother. Pharmacol.* 47,429-436.

Chen, D.J., and Nirodi, C.S. (2007). The epidermal growth factor receptor: a role in repair of radiation-induced DNA damage. *Clin. Cancer. Res.* 13, 6555-6560.

Cheng, G., Kong, D., Hou, X., Liang, B., He, M., Liang, N., Ma, S., and Liu, X. (2013). The tumor suppressor, p53, contributes to radiosensitivity of lung cancer cells by regulating autophagy and apoptosis. *Cancer Biother. Radiopharm.* 28(2),153-9.

Chipuk, J.E., and Green, D.R. (2006). Dissecting p53-dependent apoptosis. *13(6)*, 994-1002.

Choi, A.M., Ryter, S.W., and Levine, B. (2013a). Autophagy in human health and disease. *N. Engl. J. Med.* 368, 651-662.

Colston, K.W., Chander, S.K., Mackay A.G.,and Coombes, R.G. (1993). Effects of synthetic vitamin D analogues on breast cancer cell proliferation in vivo and in vitro. *Biochem. Pharmacol.* 44, 693.

Crespo, J.L., Powers, T., Fowler, B., and Hall, M.N. (2002). The TOR-controlled transcription activators GLN3, RTG1, and RTG3 are regulated in response to intracellular levels of glutamine. *Proc. Natl. Acad. Sci. USA* 99:6784-6789.

Debacq-Chainiaux, F., Erusalimsky, J.D., Campisi, J., and Toussaint, O. (2009). Protocols to detect senescence-associated beta-galactosidase (SA-beta-gal) activity, a biomarker of senescent cells in culture and in vivo. *Nat. Protoc.* 4(12), 1798-806.

Deeb, K.K., Trump, D.L., and Johnson, C.S. (2007). Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nat. Rev. Cancer.* 7(9), 684–700.

Degenhardt, K., Mathew, R., Beaudoin, B., et al. (2006). Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell.*10:51–64.

DeLuca, H.F. (2004). Overview of general physiologic features and functions of vitamin D. *Am. J. Clin. Nutr.* 80(Suppl. 6), 1689S–1696S.

Demasters, G., Di, X., Newsham, I., Shiu, R., and Gewirtz, D.A. (2006). Potentiation of radiation sensitivity in breast tumor cells by the vitamin D3 analogue, EB 1089, through promotion of autophagy and interference with proliferative recovery. *Mol. Cancer Ther.* 5(11), 2786-97.

Demasters, G.A., Gupta, M.S., Jones, K.R., Cabot, M., Wang, H., Gennings, C., Park, M., Bratland, A., Ree, A.H., and Gewirtz, D.A. (2004). Potentiation of cell killing by fractionated radiation and suppression of proliferative recovery in MCF-7 breast tumor cells by the vitamin D3 analog EB 1089. *J. Steroid Biochem. Mol. Biol.* 92, 365-374.

Dent, P., Yacoub, A., Contessa, J., Caron, R., Amorino, G., Valerie, K., Hagan, M.P., Grant, S., and Schmidt-Ullrich, R. (2003). Stress and radiation-induced activation of multiple intracellular signaling pathways. *Radiat. Res.* 159, 283-300.

Digaleh, H., Kiaei, M., and Khodagholi, F. (2013). Nrf2 and Nrf1 signaling and ER stress crosstalk: implication for proteosomal degradation and autophagy. *Cell Mol. Life Sci.* 70(24), 4681-94.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-smith, O., et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. USA.* 92(20), 9363-7.

Dutta, D., Xu, J., Kim, J.S., Dunn, W.A. Jr., and Leeuwenburgh, C. (2013). Upregulated autophagy protects cardiomyocytes from oxidative stress-induced toxicity. *Autophagy.* 9(3):328-44.

Dosoretz, D.E., Katin, M.J., Blitzer, P.H., et al.(1992). Radiation therapy in the management of medically inoperable carcinoma of the lung: results and implications for future treatment strategies. *Int. J. Radiat. Oncol. Biol. Phys.* 24(1): 3-9

Ederhardt, W., Pottgen, C., and Stuschke, M. (2006). Chemoradiation paradigm for the treatment of lung cancer. *Nature Clinical Practice Oncology.* 3, 188-199.

Eelen, G., Verlinden, L., van Camp, M., van Hummelen, P., Marchal, K., de Moor, B., Mathieu, C., Carmeliet, G., Bouillon, R., and Verstuyf, A. (2004). The effects of 1a,25-dihydroxyvitamin D3 on the expression of DNA replication genes. *J. Bone Miner. Res.* 19(1), 133–146.

Egan, D. F., Shackelford, D.B., Mihaylova, M.M., Gelino, S., Kohnz, R.A., Mair, W., Vasquez, D.S., Joshi, A., Gwinn, D.M., Taylor, R., Asara, J.M., Fitzpatrick, J., Dillin, A., Viollet, B., Kundu, M., Hansen, M., and Shaw, R.J. et al. (2011). Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science.* 331,456–461.

Elmore S. (2007). Apoptosis: A review of programmed cell death. *Toxicology Pathology.*

Evan GI, d'Adda di Fagagna F. (2009) Cellular senescence: hot or what? *Curr. Opin. Genet. Dev.* 19, 25–31.

Falck, J., Coates, J., and Jackson, S.P. (2005). Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. *Nature*. 434(7033):605-11.

Feng, Z., Zhang, H., Levine, A.J., and Jin, S. (2005). The coordinate regulation of the p53 and mTOR pathways in cells. *Proc. Natl. Acad. Sci. U S A*. 102(23), 8204-9.

Feng, Z., Hu, W., de Stanchina, E., Teresky, A.K., Jin, S., Lowe, S., and Levine, A.J. (2007). The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. *Cancer Res*. 67(7):3043-53.

Flaig, T.W., Barqawi, A., Miller, G., Kane, M., Zeng, C., Crawford, E.D., and Glode, L.M. (2006). A phase II trial of dexamethasone, vitamin D, and carboplatin in patients with hormone-refractory prostate cancer. *Cancer*. 107(2), 266–274.

Fogarty, S., Hawley, S.A., Green, K.A., Saner, N., Mustard, K.J., and Hardie, D.G. (2010). Calmodulin-dependent protein kinase kinase-beta activates AMPK without forming a stable complex: synergistic effects of Ca²⁺ and AMP. *Biochem. J*. 426, 109–118.

Funakoshi, T., Matsuura, A., Noda, T., and Ohsumi, Y. (1997). Analyses of APG13 gene involved in autophagy in yeast, *Saccharomyces cerevisiae*. *Gene*. 192, 207–13.

Gadbois, D.M., Crissman, H.A., Nastasi, A., Habbersett, R., Wang, S.K., Chen, D., Lehnert, B.E. (1996). Alterations in the progression of cells through the cell cycle after exposure to alpha particles or gamma rays. *Radia. Res*. 146(4): 414-424.

Ganley, I.G., Lam du, H., Wang, J., Ding, X., Chen, S., and Jiang, X. (2009). ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. *J. Biol. Chem*. 284,12297–305.

Gauden, S., Ramsay, J., and Tripcony, L. (1995). The curative treatment by radiotherapy alone of stage I non-small cell carcinoma of the lung. *Chest*. 108(5):1278-82,

Gewirtz, D.A., Gupta, M.S., and Sundaram, S. (2002). Vitamin D3 and vitamin D3 analogues as an adjunct to cancer chemo-therapy and radiotherapy. *Curr Med Chem Anticancer Agents*. 2(6), 683-90.

Gewirtz, D.A., Holt, S.E., and Elmore, L.W. (2008). Accelerated senescence: an emerging role in tumor cell response to chemotherapy and radiation. *Biochem. Pharmacol*. 76, 947–957.

Gewirtz, D.A. (2009). Autophagy, senescence and tumor dormancy in cancer therapy. *Autophagy*. 5,1232–4.

Gewirtz, D.A. (2014). The four faces of autophagy: implications for cancer therapy. *Cancer Res.* 74(3), 647-51.

Giovannucci, E. (2008). Vitamin D status and cancer incidence and mortality. *Adv. Exp. Med. Biol.* 624, 31–42.

Gwinn, D. M., Shackelford, D.B., Egan, D.F., Mihaylova, M.M., Mery, A., Vasquez, D.S., Turk, B.E., and Shaw, R.J. (2008). AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol. Cell.* 30,214–226.

Hall, E., and Giaccia, A. (2006). *Radiobiology for Radiologist* Lippincott Williams and Wilkins.

Hamamoto, Y., Kataoka, M., Nogami, N., Kozuki, T., Kato, Y., Shinohara, S., and Shinkai, T. (2012). Factors affecting survival time after recurrence of non-small-cell lung cancer treated with concurrent chemotherapy. *Jpn. J. Radiol.* 30(3), 249-54.

Hansdottir, S., Monick, M.M., Hinde, S.L., Lohan, N., Look, D.C., and Hunninghake, G.W. (2008). Respiratory epithelial cells convert inactive vitamin D to its active form: potential effects on host defense. *J. Immunol.* 181(10), 7090-7099.

Hanahan, D. and Weinberg, R.A. (2000). The hallmarks of cancer. *Cell.* 100(1), 57-70.

Hawley, S.A., Boudeau, J., Reid, J.L., Mustard, K.J., Udd, L., Makela, T.P., Alessi, D.R., and Hardie, D.G. (2003). Complexes between the LKB1 tumor suppressor, STRADalpha/beta and MO25alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J. Biol.* 2, 28.

Hara, T., Takamura, A., Kishi, C., Lemura, S., Natsume, T., Guan, J.L., and Mizushima, N. (2008). FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. *J.Cell Biol.* 181(3), 497-510.

Hardie, D.G., Ross, F.A., and Hawley, S.A. (2012). AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat. Rev. Mol. Cell Biol.* 13(4), 251-62.

Hayflick, L. (1965). The limited in vitro lifetime of human diploid cell strains. *Exp. Cell Res.* 37, 614–636.

Hayflick, L. and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. 25:585-621.

Hershberger, P.A., Yu, W.D., Modzelewski, R.A., Rueger, R.M., Johnson, C.S., and Trump, D.L. (2001). Calcitriol (1,25-dihydroxycholecalciferol) enhances paclitaxel antitumor activity in vitro and in vivo and accelerates paclitaxel-induced apoptosis. *Clin. Cancer Res.* 7, 1043-1051.

Hillyer, R.L., Srinivasin, P., Joglekar, M., Sikes, R.A., van Golen, K.L., and Nohe, A. (2012). Differential effects of vitamin D treatment on inflammatory and non-inflammatory breast cancer cell lines. *Clin. Exp. Metastasis.* 29(8):97-9.

Hollomon, M.G., Gordon, N., Santiago-O'Farrill, J.M., Kleinerman, E.S. (2013). Knockdown of autophagy-related protein 5, ATG5, decreases oxidative stress and has an opposing effect on camptothecin-induced cytotoxicity in osteosarcoma cells. *BMC Cancer* 13:500.

Holick, M.F. (2007). Vitamin D deficiency. *N Engl J Med.* 357(3), 266- 281.

Honscheid, P., Datta, K., and Muders, M.H. (2014). Autophagy: Detection, regulation and its role in cancer and therapy response. *Int. J. Radiat. Biol.* 90(8), 628-35.

Hosokawa, N., Sasaki, T., Iemura, S., Natsume, T., Hara, T., and Mizushima, N. (2009a). Atg101, a novel mammalian autophagy protein interacting with Atg13. *Autophagy.* 5, 973–9.

Hosokawa, N., Hara, T., Kaizuka, T., Kishi, C., Takamura, A., Miura, Y., Iemura, S., Natsume, T., Takehana, K., Yamada, N., Guan, J.L., Oshiro, N., and Mizushima, N. (2009b). Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol. Biol. Cell.* 20,1981–91.

Hoyer-Hansen, M., Bastholm, L., Szyniarowski, P., Campanella, M., Szabadkai, G., Farkas, T., Bianchi, K., Fehrenbacher, N., Elling, F., Rizzuto, R., Mathiasen, I.S., and Jaattela, M. (2007). Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. *Mol. Cell.* 25(2), 193–205.

Hoyer-Hansen, M., Bastholm, L., Mathiasen, I.S., Elling, F., and Jaattela, M. (2005). Vitamin D analog EB1089 triggers dramatic lysosomal changes and Beclin 1-mediated autophagic cell death. *Cell Death Differ.* 12(10):1297–1309.

Hurley, R.L., Anderson, K.A., Franzone, J.M., Kemp, B.E., Means, A.R., and Witters, L.A. (2005). The Ca²⁺/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. *J. Biol. Chem.* 280, 29060–29066.

Igney, F.H., and Krammer, P.H. (2002). Death and Anti-death: Tumour resistance to apoptosis. *Nat.Rev.Cancer*. 2(4), 277-88.

Ilahi, M., Armas, L.A., and Heaney, R.P. (2008). Pharmacokinetics of a single, large dose of cholecalciferol. *Am. J. Clin. Nutr.* 87(3):688-91.

Ingraham, B.A., Bragdon, B., and Nohe, A. (2008). Molecular basis of the potential of vitamin D to prevent cancer. *Curr. Med. Res. Opin.* 24(1), 139-49.

Inoki, K., Kim, J., and Guan, K.L. (2012). AMPK and mTOR in cellular energy homeostasis and drug targets. *Annu. Rev. Pharmacol. Toxicol.* 52, 381-400.

Inoki, K., Zhu, T., and Guan, K.L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. *Cell*. 115,577–590.

James, C.F. (2008). Molecular actions of vitamin D contributing to cancer prevention. *Mol. Aspects Med.* 29(6), 388-396.

Jeong, Y., Xie, Y., Lee, W., Bookout, A.L., Girard, L., Raso, G., Behrens, C., Wistuba II, Gadzar, A.F., Minna, J.D. et al. (2012). Research Resource: Diagnostic and Therapeutic potential of nuclear receptor expression in lung cancer. *Mol Endocrinol.* 26(8):1443-1454.

Johnson, D.H., Blot, W.J., Carbone, D.P., et al. (2008). Cancer of the lung: non-small cell lung cancer and small cell lung cancer. In: Abeloff MD, Armitage JO, Niederhuber JE, et al., eds. *Abeloff's Clinical Oncology*. 4th ed. Philadelphia, Pa: Elsevier Churchill Livingstone; chap 76.

Jones, K.R., Elmore, L.W., Jackson-Cook, C., Demasters, G., Povirk, L.F., Holt, S.E., and Gewirtz, D.A. (2005). p53-dependent accelerated senescence induced by ionizing radiation in breast tumor cells. *Int. J. Radiat. Biol.* 81(6), 1686-700.

Jung, C.H., Ro, S.H., Cao, J., Otto, N.M., and Kim, D.H. (2010). mTOR regulation of autophagy. *FEBS left.* 584(7),1287-95.

Jung, C.H., Jun, C.B., Ro, S.H., Kim, Y.M., Otto, N.M., Cao, J., Kundu, M., and Kim, D.H. (2009). ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol. Biol. Cell.* 20,1992–2003.

Kabeya, Y., Noda, N.N., Fujioka, Y., Suzuki, K., Inagaki, F., and Ohsumi, Y. (2009). Characterization of the Atg17- Atg29-Atg31 complex specifically required for starvation-induced autophagy in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* 389,612–5.

Kabeya, Y., Kamada, Y., Baba, M., Takikawa, H., Sasaki, M., and Ohsumi, Y. (2005). Atg17 functions in cooperation with Atg1 and Atg13 in yeast autophagy. *Mol. Biol. Cell.* 16, 2544–53.

- Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M., and Ohsumi, Y. (2000). Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J. Cell Biol.* 150, 1507–13.
- Kang, R., Zeh, H.J., Lotze, M.T., and Tang, D. (2011). The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ.* 18(4), 571–580.
- Kaplan, K., Miyamoto, R., Levine, B.R., Egol, K.A., and Zuckerman, J.D. (2008). Surgical management of hip fractures: an evidence-based review of the literature. II: intertrochanteric fractures. *J. Am. Acad. Orthop. Surg.* 16, 665-673.
- Kawamata, T., Kamada, Y., Kabeya, Y., Sekito, T., and Ohsumi, Y. (2008). Organization of the pre-autophagosomal structure responsible for autophagosome formation. *Mol. Biol. Cell.* 19, 2039–50.
- Kazgan, N., Williams, T., Forsberg, L.J., and Brenman, J.E. (2010). Identification of a nuclear export signal in the catalytic subunit of AMP-activated protein kinase. *Mol. Biol. Cell.* 21, 3433-3442.
- Kenzelmann Broz, D., Spano Mello, S., Bieging, K.T., Jiang, D., Dusek, R.L., Brady, C.A., Sidow, A., and Attardi, L.D. (2013). Global genomic profiling reveals an extensive p53-regulated autophagy program contributing to key p53 responses. *Genes Dev.* 27(9), 1016-31.
- Kihara, A., Kabeya, Y., Ohsumi, Y., and Yoshimori, T. (2001). Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. *EMBO Rep.* 2(4), 330–335.
- Kim, J., Kundu, M., Viollet, B., and Guan, K.L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat. Cell Biol.* 13,132–141.
- Kim, K.W., Moretti, L., Mitchell, L.R., Jung, D.K., and Lu B. (2010). Endoplasmic reticulum stress mediates radiation-induced autophagy by perk-eIF2alpha in caspase-3/7-deficient cells. *Oncogene.* 29(22), 3241-51.
- Kim, K.W., Speirs, C.K., Jung, D.K., and Lu, B. (2011). The zinc ionophore PCI-5002 radiosensitizes non-small cell lung cancer cells by enhancing autophagic cell death. *J. Thorac. Oncol.* 6(9):1542-52.
- Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., and Ohsumi, Y. (2000). The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *J. Cell Biol.* 151, 263-276.

Kirkland R. A., Adibhatla R. M., Hatcher J. F., Franklin J. L. (2002). Loss of cardiolipin and mitochondria during programmed neuronal death: evidence of a role for lipid peroxidation and autophagy. *Neuroscience*. 115:587–602.

Klionsky, D.J. et al. (2012). Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy*. 8(4), 455-544.

Ko, A., Kanehisa, A., Martins, I., Senovilla, L., Chargari, C., Dugue, D., Marino, G., Kepp, O., Michaud, M., Perfettini, J.L., Kroemer, G., and Deutsch, E. (2014). Autophagy inhibition radiosensitizes in vitro, yet reduces radioresponses in vivo due to deficient immunogenic signaling. *Cell Death Differ*. 21, 92–9.

Koohdani, F., Baghdadchi, J., Sasani, F., Mohammad, K. and Mehdipour, P. (2008). Effects of vitamin D in lung, stomach, esophagus and testis tissues following administration of urethane in Balb/c mice. *Saudi Med. J*. 29(3), 340-344.

Krishnan, A.V., Trump, D.L., Johnson, C.S., and Feldman, D. (2010). The role of vitamin D in cancer prevention and treatment.. *Endocrinol. Metab. Clin. N. AM*. 39, 401-418.

Krishnan, A.V., Trump, D.L., Johnson, C.S., and Feldman, D. (2012). The role of vitamin D in cancer prevention and treatment. *Rheum. Dis. Clin. North Am*. 38(1), 161-78.

Kroemer, G., and Levine, B. (2008). Autophagic cell death: the story of a misnomer. *Nat. Rev. Mol. Cell Biol*. 9(12): 1004-10.

Kuma, A., Matsui, M., and Mizushima, N. (2007). LC3, an autophagosome marker, can be incorporated into protein aggregates independent of autophagy: caution in the interpretation of LC3 localization. *Autophagy*. 3(4), 323-8.

Kurosaka, K., Takahashi, M., Watanabe, N., and Kobayashi, Y. (2003). Silent cleanup of very early apoptotic cells by macrophages. *J. Immunol*. 171, 4672–9.

Lee, A.C., Fenster, B.E., Ito, H., Takeda, K., Bae, N.S., Hirai, T., Yu, Z.X., Ferrans, V.J., Howard, B.H., Finkel, T. (1999). Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. *J. Biol. Chem*. 274, 7936–7940.

Lee, B.Y., J.A. Han, J.S. Im, A. Morrone, K. Johung, E.C. Goodwin, W.J. Kleijer, D. DiMaio, and E.S. Hwang. (2006). Senescence-associated betagalactosidase is lysosomal beta-galactosidase. *Aging Cell*. 5,187–195.

Lee, J.W., Park, S., Takahashi, Y., and Wang, H.G. (2010). The association of AMPK with ULK1 regulates autophagy. *PLoS One*. 5, e15394.

Lee, K.H., Li, M., Michalowski, A.M., Zhang, X., Liao, H., Chen, L., Xu, Y., Wu, X., and Huang, J. (2010). A genomewide study identifies the Wnt signaling pathway as a major target of p53 in murine embryonic stem cells. *Proc. Natl. Acad. Sci*. 107, 69–74.

Levine, A.J., Hu, W., and Feng, Z. (2006). The p53 pathway: what questions remain to be explored? *Cell Death Differ.* 13(6), 1027-36.

Levine, B., and Abrams, J. (2008). p53: The Janus of autophagy? *Nat. Cell Biol.* 10(6): 637-9.

Levine, B., Mizushima, N., and Virgin, H.W. (2011). Autophagy in immunity and inflammation. *Nature.* 469, 323-335.

Li, D.D., Sun, T., Wu, X.Q., Chen, S.P., Deng, R., Jiang, S., Feng, G.K., Pan, J.X., Zhang, X.S., Zeng, Y.X., Zhu, X.F. (2012). The inhibition of autophagy sensitises colon cancer cells with wildtype p53 but not mutant p53 to topotecan treatment. *PLoS One* 7(9):e45058.

Li, M., He, Y., Dubois, W., Wu, X., Shi, J., and Huang, J. (2012). Distinct regulatory mechanisms and functions for p53-activated and p53-repressed DNA damage response genes in embryonic stem cells. *Mol. Cell* 46, 30–42.

Light, B.W., Yu, W.D., McElwain, M.C., Russell, D.M., Trump, D.L., and Johnson, C.S. (1997). Potentiation of cisplatin antitumor activity using a vitamin D analogue in a murine squamous cell carcinoma model system. *Cancer Res.* 57, 3759-3764.

Liu B, Cheng Y, Liu Q, Bao JK, Yang JM (2010) Autophagic pathways as new targets for cancer drug development. *Acta Pharmacol Sin* 31:1154–1164

.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P., Hall, M.N. (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol. Cell.* 10(3), 457–468.

Lorin, S., Hamaï, A., Mehrpour, M., and Codogno, P. (2013). Autophagy regulation and its role in cancer. *Semin Cancer Biol.* 2013. *Cancer Biother. Radiopharm.* 28(2),152-9.

Lu, Z., Luo, R.Z., Lu, Y., Zhang, X., Yu, Q., Khare, S., Kondo, S., Kondo, Y., Yu, Y., Mills, G.B., Liao, W.S., and Bast, R.C.Jr. (2008). The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells. *J Clin Invest.* 118, 3917–29.

Luo, H., Wang, L., Schulte, B.A., Yang, A., Tang, S., and Wang, G.Y. (2013). Resveratrol enhances ionizing radiation-induced premature senescence in lung cancer cells. *Int. J. Oncol.* 43(6): 1999-2006.

Maiuri, M.C., Le Toumelin, G., Criollo, A., Rain, J.C., Gautier, F., Juin, P., Tasdemir, E., Pierron, G., Maruyama, R., Aoki, F., Toyota, M., Sasaki, Y., Akashi, H., Mita, H., Suzuki, H., Akino, K., Ohe-Toyota, M., Maruyama, Y., Tatsumi, H., Imai, K., Shinomura, Y., and

Tokino, T. (2006). Comparative genome analysis identifies the vitamin D receptor gene as a direct target of p53-mediated transcriptional activation. *Cancer Res.* 66(9), 4574-83.

Maruyama, R., Akoi, F., Toyota, M., Sasaki, Y., Akashi, H., Mita, H., Suzuki, H., Akino, K., Ohe-Toyota, M., Maruyama, Y., Tatsumi, H., Imai, K., Shinomura, Y., Tokino, T. (2006). Comparative genome analysis identifies the vitamin D receptor gene as a direct target of p53-mediated transcriptional activation. *Cancer Res.* 66(9):4574-83.

Matsuura, A., Tsukada, M., Wada, Y., and Ohsumi, Y. (1997). Apg1p, a novel protein kinase required for the autophagic process in *Saccharomyces cerevisiae*. *Gene.* 192,245–50.

McGuire, M.F., Trump, D.L., and Johnson, C.S. (2001). Vitamin D3-induced apoptosis of murine squamous cell carcinoma cells: selective induction of caspase-dependent MEK cleavage and up-regulation of MEKK-1. *J. Biol. Chem.* 276(28), 26365-73.

Mercer, C.A., Kaliappan, A., and Dennis, P.B. (2009). A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy. *Autophagy.* 5, 649–62.

Michaud, M., Martins, I., Sukkurwala, A.Q., Adjemian, S., Ma, Y., Pellegatti, P., Shen, S., Kepp, O., Scoazec, M. et al. (2011). Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. *Science.* 334, 1573–7.

Mihaylova, M.M., and Shaw, R.J. (2011). The AMPK signaling pathway coordinates cell growth, autophagy and metabolism. *13(9)*, 1016-23.

Milczarek, M., Chodynski, M., Filip-psurska, B., Martowicz, A., Krupa, M., Krajewski, K., Kutner, A., and Wietrzyk, J. (2013). Synthesis and biological activity of Diastereomeric and Geometric analogs of calcipotriol, PRI-2201 and PRI-2205, against human HL-60 leukemia and MCF-7 breast cancer cells. *5(4)*, 1355-78.

Mizushima, N. (2007). Autophagy: process and function. *Genes Dev.* 21(22):2861-73.

Mizushima, N., Yoshimori, T., and Levine, B. (2010). Methods in mammalian autophagy research. *Cell.* 140(3), 313-26.

Morselli, E., Tasdemir, E., Maiuri, M.C., Galluzzi, L., Kepp, O., Criollo, A., Vicencio, J.M., Soussi, T., Kroemer, G. (2008). Mutant p53 protein localized in the cytoplasm inhibits autophagy. *Cell Cycle.* 7(19), 3056-61.

Muindi, J.R., Johnson, C.S., Trump, D.L., Christy, R., Engler, K.L., and Fakih, M.G. (2009). A phase I and pharmacokinetics study of intravenous calcitriol in combination with oral dexamethasone and gefitinib in patients with advanced solid tumors. *Cancer Chemotherapy and Pharmacology.* 65(1), 33–40.

Mu"ller O, Sattler T, Flo"tenmeyer M, Schwarz H, Plattner H, Mayer A (2000) Autophagic tubes: vacuolar invaginations involved in lateral membrane sorting and inverse vesicle budding. *J Cell Biol* 151:519–528

Murrow, L., and Debnath, J. (2013). Autophagy as a stress-response and qualitycontrol mechanism: implications for cell injury and human disease. *Annu. Rev. Pathol.* 8,105–37.

Nagpal, S., Na, S., and Rathnachalam, R. (2005). Noncalcemic actions of vitamin D receptor ligands. *Endocr. Rev.* 26(5), 662–687.

Narita, M., N"unez, S., Heard, E., Narita, M., Lin, A.W., Hearn, S.A., Spector, D.L, Hannon, G.J., and Lowe, S.W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell.* 113,703–716.

Narita, M., Young, A.R., and Narita, M. (2009). Autophagy facilitates oncogene-induced senescence. *Autophagy* 5, 1046-1047.

National Cancer Institute, T. (2014a). Lung Cancer. 2013

National Cancer Institute, T. (2007b). Radiation therapy and You: Support for people with cancer. 2014.

National Cancer Institute, T. (2010a). Radiation therapy for cancer. 2014.

Nemere, I., Safford, S.E., Rohe, B., DeSouza, M.M., and Farach-Carson, M.C. (2004). Identification and characterization of 1,25D-3-membraneassociated rapid response, steroid (1,25D3-MARRS)- binding protein. *J Steroid Biochem Mol Biol* 89-90(0), 281-285.

Noda, T., and Ohsumi, Y. (1998). Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J. Biol. Chem.* 273, 3963–6.

Norton, R. and O'Connell, M.A. (2012). Vitamin D: Potential in the prevention and treatment of lung cancer. *Anticancer Res.* 32(1), 211-21.

Oakhill, J.S., Steel, R., Chen, Z.P., Scott, J.W., Ling, N., Tam, S., and Kemp, B.E. (2011). AMPK is a direct adenylate charge-regulated protein kinase. *Science.* 332, 1433–1435.

Pan, J., Song, E., Cheng, C., Lee, M.H., Yeung, S.C. (2009). Farnesyl-transferase inhibitors-induced autophagy:alternative mechanisms? *Autophgy.* 5:129-131.

Park, W.H., Seol, J.G., Kim, E.S., Jung, C.W., Lee, C.C., Binderup, L., Koeffler, H.P., Kim, B.K., Lee, Y.Y. (2000). Cell cycle arrest induced by the vitaminD3 analog EB 1089

in NCI-H929 myeloma cells is associated with induction of cyclin-dependent kinase inhibitor p27. *Exp. Cell. Res.* 1:254(2):279-86.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D., and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell.* 122(6), 927–939.

Polar, M.K., Gennings, C., Park, M., Gupta, M.S., and Gewirtz, D.A. (2003). Effect of vitamin D3 analog ILX 23-7553 on apoptosis and sensitivity to fractionated radiation in breast tumor cells and normal fibroblasts. *Cancer Chemother. Pharmacol.* 51(5), 415-21.

Porojnicu, A.C., Dahlback, A., and Moan, J. (2008). Sun exposure and cancer survival in Norway: changes in the risk of death with season of diagnoses and latitude. *Adv. Exp. Med. Biol.* 624, 43-54.

Prudencio, J., Akutsu, N., Benlimame, N., Wang, T., Bastien, Y., Lin, R., Black, M.J., Alaoui-Jamali, M.A., and White, J.H. (2001). Action of low calcemic 1 α ,25-dihydroxyvitamin D3 analogue EB1089 in head and neck squamous cell carcinoma. *J. Natl. Cancer inst.* 93(10), 745-53.

Radermacher, J., Diesel, B., Seifert, M., Tilgen, W., Reichrath, J.R., Fischer, U. and Meese, E. (2006). Expression analysis of CYP27B1 in tumor biopsies and cell cultures. *Anticancer Res* 26(4A), 2683- 2686.

Ramnath, N., Daignault-Newton, S., Dy, G.K., Muindi, J.R., Adjei, A., Elingrod, V.L., Kalemkerian, G.P., Cease, K.B., Stella, P.J., Brenner, D.E., Troeschel S., Johnson, C.S., and Trump, D.L. (2013). A phase I/II pharmacokinetic and pharmacogenomics study of calcitriol in combination with cisplatin and docetaxel in advanced non-small-cell lung cancer. *Cancer Chemother Pharmacol.* 71(5), 1173-82.

Ramnath, N., Kim, S., and Christenden, P.J. (2011). Vitamin D and lung cancer. *Expert Rev. Respir. Med.* 5(3), 305-9.

Reichrath, J., Reichrath, S., Heyne, K., Vogt, T., and Roemer, K. (2014). Tumor suppression in skin and other tissues via cross-talk between vitamin D and p53-signaling. *Front Physiol.* 5:166

Richard, C.L., Farach-Carson, M.C., Rohe, B., Nemere, I. and Meckling, K.A. (2010). Involvement of 1,25D3-MARRS (membrane associated, rapid response steroid-binding), a novel vitamin D receptor, in growth inhibition of breast cancer cells. *Exp. Cell. Res.* 316(5), 695-703.

Roach, P.J. (2011). AMPK -> ULK1 -> autophagy. *Mol. Cell. Biol.* 31(15), 3082-4.

Rodier, F. and Campisi, J. (2011). Four faces of cellular senescence. *J. Cell Biol.* 192(4), 547-56.

Samari, H.R. and Seglen, P.O. (1998). Inhibition of hepatocytic autophagy by adenosine, aminoimidazole-4-carboxamide riboside, and N6-mercaptopurine riboside. Evidence for involvement of AMP-activated protein kinase. *J. Biol. Chem.* 273, 23758–23763.

Sancar, A., Lindsey-Boltz, L.A., Unsal-Kacmaz, K., and Linn, S. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu. Rev. Biochem.* 73:39-85.

Sanli, T., Rashid, A., Liu, C., Harding, S., Bristow, R.G., Cutz, J.C., Singh, G., Wright, J., and Tsakiridis, T. (2010). Ionizing radiation activates AMP-activated kinase (AMPK): a target for radiosensitization of human cancer cells. *Int. J. Radiat. Oncol. Biol. Phys.* 78(1), 221-9.

Sarkar, S. (2013). Regulation of autophagy by mTOR-dependent and mTORindependent pathways: autophagy dysfunction in neurodegenerative diseases and therapeutic application of autophagy enhancers. *Biochem. Soc. Trans.* 41,1103–30.

Savarino, A., Lucia, M.B., Giordano, F., and Cauda, R. (2006). Risks and benefits of chloroquine use in anticancer strategies. *Lancet. Oncol.* 7(10), 792-3.

Scott, R.C., Schuldiner, O., and Neufeld, T.P. (2004). Role and regulation of starvation-induced autophagy in the *Drosophila* fat body. *Dev. Cell.* 7,167–78.

Scherz-Shouval, R., Shvets, E., Fass, E., Shorer, H., Gil, L., Elazar, Z. (2007). Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *EMBO J.* 26:1749–1760.

Shaffer, P.L., and Gewirth, D.T. (2002). Structural basis of VDR-DNA interactions on direct repeat response elements. *EMBO J.* 21(9), 2242–2252

Shaw, R.J., Kosmatka, M., Bardeesy, N., Hurley, R.L., Witters, L.A., DePinho, R.A., and Cantley, L.C. (2004). The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc. Natl. Acad. Sci. U S A.* 101, 3329–3335.

Shay, J.W., and Roninson, I.B. (2004). Hallmarks of senescence in carcinogenesis and cancer therapy. *Oncogene.* 23, 2919–2933.

Shen, S., Kepp, O., Michaud, M., Martins, I., Minoux, H., Metivier, D., Maiuri, M.C., Kroemer, R.T., Kroemer, G. (2011). Association and dissociation of autophagy , apoptosis, and necrosis by systematic chemical study. *Oncogene.* 30(45):4544-56.

Siegel, R., Naishadham, D., and Jemal, A. (2012). Cancer statistics. *CA Cancer J Clin* 62(1), 10-29.

Sinha, S., and Levine, B. (2008). The autophagy effector Beclin 1: a novel BH3-only protein. *Oncogene*. 27 Suppl 1, S137–S148.

Song, Y., Peng, X., Porta, A., Takanaga, H., Peng, J.B., Hediger, M.A., Fleet, J.C., and Christakos, S. (2003). Calcium transporter 1 and epithelial calcium channel messenger ribonucleic acid are differentially regulated by 1,25 dihydroxyvitamin D3 in the intestine and kidney of mice. *Endocrinology*. 144(9), 3885–3894.

Sperber, K., Chiang, G., Chen, H., Ross, W., Chusid, E., Gonchar, M., Chow, R., and Liriano, O. (1997). Comparison of hydroxychloroquine with zidovudine in asymptomatic patients infected with human immunodeficiency virus type 1. *Clin. Ther.* 19(5):913-23.

Spitz, D.R., Azzam, E.I., Li, J.J., and Gius, D. (2004). Metabolic oxidation /reduction reactions and cellular responses to ionizing radiation: a unifying concept in stress response biology. *Cancer metastasis Rev.* 23, 311-322.

Stambolsky, P., Tabach, Y., Fontemaggi, G., Weisz, L., Maor-Aloni, R., Shiff, I., Kogan, I., Shay, M., Kalo, E., Blandino, G., Simon, I., Oren, M., and Rotter, V. (2010). Modulation of vitamin D3 response by cancer-associated p53. *Cancer cell*. 17(3), 273-285.

Straub, M., Bredschneider, M., and Thumm, M. (1997). AUT3, a serine/threonine kinase gene, is essential for autophagocytosis in *Saccharomyces cerevisiae*. *J. Bacteriol.* 179, 3875–83.

Sui, X., Chen, R., Wang, Z., Huang, Z., Kong, N., Zhang, M., Han, W., Lou, F., Yang, J., Zhang, Q., Wang, X., He, C., and Pan, H. (2013). Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. *Cell Death Dis.* 4:e838

Sundaram, S., and Gewirtz, D.A. (1999). The vitamin D3 analog EB 1089 enhances the response of human breast tumor cells to radiation. *Radiat. Res.* 152(5),479-86.

Sundaram, S., Sea, A., Feldman, S., Strawbridge, R., Hoopes, P.J., Demidenko, E., Binderup, L., and Gewirtz, D.A. (2003). The combination of a potent vitamin D3 analog, EB 1089, with ionizing radiation reduces tumor growth and induces apoptosis of MCF-7 breast tumor xenografts in nude mice. *Clin. Cancer Res.* 9(6), 2350-6.

Sun, J.G., Liao, R.X., Zhang, S.X., Duan, Y.Z., Zhuo, W.L., Wang, X.X., Wang, Z.X., Li, D.Z., and Chen, Z.T. (2011). Role of inhibitor of apoptosis protein Livin in radiation resistance in non-small cell lung cancer. *Cancer Biother. Radiopharm.* 26(5): 585-92.

Sun, Q., Fan, W., and Zhong, Q. (2009). Regulation of Beclin 1 in autophagy. *Autophagy*. 5(5), 713–716.

Tapper, H. and Sundler, R. (1995). Bafilomycin A1 inhibits lysosomal, phagosomal, and plasma membrane H(+)-ATPase and induces lysosomal enzyme secretion in macrophages. *J. Cell physiol.* 163(1),137-44.

Tasdemir, E., Maiuri, M.C., Galluzzi, L., Vitale, I., Djavaheri- Mergny, M., D'Amelio, M., Criollo, A., Morselli, E., Zhu, C., et al. (2008a). Regulation of autophagy by cytoplasmic p53. *Nat. Cell Biol.* 10:676-87.

Tasdemir, E., Maiuri, M.C., Orhon, I., Kepp, O., Morselli, E., Criollo, A., and Kroemer, G. (2008b). p53 represses autophagy in a cell cycle-dependent fashion. *Cell Cycle*. 7(19):3006-11

Troulinaki, K., Tavernarakis, N., Hickman, J.A., Geneste, O., and Kroemer, G. (2007). Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. *EMBO J.* 26(10), 2527–2539.

Tsakonas, E., Fitzgerald, A.A., Fitzcharles, M.A., Cividino, A., Thorne, J.C., M'Seffar, A., Joseph, L., Bombardier, C., and Esdaile, J.M. (2000). Consequences of delayed therapy with second-line agents in rheumatoid arthritis: a 3 year follow up on the hydroxychloroquine in early rheumatoid arthritis (HERA) study. *J. Rheumatol.* 67(7):3043-53.

Uttenweiler, A., Schwarz, H., and Mayer, A. (2005) Microautophagic vacuole invagination requires calmodulin in a Ca²⁺ Independent function. *J Biol Chem* 280:33289–33297.

Uziel, T., Lerenthal, Y., Moyal, L., Andegeko, Y., Mittelman, L., and Shiloh, Y. Recruitment of MRN complex for ATM activation by DNA damage. *EMBO J.* 22(20):5612-21.

Vanoirbeek, E., Krishnan, A., Eelen, G., Verlinden, L., Bouillon, R., Feldman, D., and Verstuyf, A. (2011). The anticancer and anti-inflammatory actions of 1,25(OH)₂D₃. *Best Pract. Res. Clin. Endocrinol. Metab.* 25(4), 593-604.

Van Leeuwen, J.P.T.M., and Pols, H.A. (2005). Vitamin D:Cancer and differentiation in vitamin D, (Burlington, M.A: Elsevier Academic Press).

Van Kaick, G., and Delorme, S. (2008). Therapy induced effects in normal tissue. *Radiologe* 48(9), 871-80.

Vazquez-Martin, A., Corominas-Faja, B., Oliveras-Ferraros, C., Cufi, S., Venezia, N.D., and Menendez, J.A. (2013). Serine 79- phosphorylated acetyl-CoA carboxylase, a

downstream target of AMPK, localizes to the mitotic spindle poles and the cytokinesis furrow. *Cell Cycle*. 12(10),1639-1641.

Vazquez G, de Boland AR, and Boland R. (1997). Stimulation of Ca²⁺ release-activated Ca²⁺ channels as a potential mechanism involved in non-genomic 1,25(OH)₂-vitamin D₃-induced Ca²⁺ entry in skeletal muscle cells. *Biochem. Biophys. Res. Commun.* 239(2):562-5.

Wang, J., Engle, S., and Zhang, Y. (2011). A new in vitro system for activating the cell cycle checkpoint. *Cell Cycle*. 10(3):500-6.

Wang, J., Lian, H., Zhao, Y., Kauss, M.A., and Spindel, S. (2008). Vitamin D₃ induces autophagy of human myeloid leukemia cells. *J Biol Chem*. 283(37), 25596–25605.

Wang, Q., Yang, W., Uytingco, M.S., Christakos, S., and Wieder, R. (2000). 1,25-Dihydroxyvitamin D₃ and all-trans-retinoic acid sensitize breast cancer cells to chemotherapy-induced cell death. *Cancer Res*. 60, 2040-2048.

Wang, R.C., Wei. Y., An. Z., Zou, Z., Xiao, G., Bhagat, G., White, M., Reichelt, J., Levine, B. (2012). Akt mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation. *Science*. 338, 956-959

Wang, Y., Singh, R., Massey, A.C., Kane, S.S., Kaushik, S., Grant, T., et al. (2008). Loss of macroautophagy promotes or prevents fibroblast apoptosis depending on the death stimulus. *J Biol Chem*. 283:4766–4777.

Wang, Z., Wilson, W.A., Fujino, M.A., and Roach, P.J. (2001). Antagonistic controls of autophagy and glycogen accumulation by Snf1p, the yeast homolog of AMP-activated protein kinase, and the cyclin-dependent kinase Pho85p. *Mol. Cell. Biol*. 21, 5742–5752.

Ward, J.F. (1988) DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability. *Prog. Nucleic Acid Res. Mol. Biol*. 35, 95–125.

Watson, S.P., McNally, J., Shipman, L.J., and Godfrey, P.P. (1988). The action of the protein kinase C inhibitor, staurosporine, on human platelets. Evidence against a regulatory role for protein kinase C in the formation of inositol triphosphate by thrombin. *Biochem J*. 249, 345-350.

Wei, C.L., Wu, Q., Vega, V.B., Chiu, K.P., Ng, P., Zhang, T., Shahab, A., Yong, H.C., Fu, Y., Weng, Z., Liu, J., Zhao, X.D., Chew, J.L., Lee, Y.L., et al. (2006). A global map of p53 transcription-factor binding sites in the human genome. *Cell*. 124, 207-219.

Wietrzyk, J., Nevozhay, D., Filip, B., Milczarek, M., and Kutner, A. (2007). The antitumor effect of lowered doses of cytostatics combined with new analogs of vitamin D in mice.

Anticancer Res. 27(5A), 3387-3398.

Wintner, L.M., Giesinger, J.M., Zabernigg, A., Sztankay, M., Meraner, V., Pall, G., Hilbe, W., and Holzner, B. (2013). Quality of life during chemotherapy in lung cancer patients: results across different treatment lines. *Br. J. Cancer*. 109(9), 2301-8.

Wilson, E.N., Bristol, M.L., Di, X., Maltese, W.A., Koterba, K., Beckman, M.J., and Gewirtz, D.A. (2011). A switch between cytoprotective and cytotoxic autophagy in the radiosensitization of breast tumor cells by chloroquine and vitamin D. *Horm Cancer*. 2(5),272-85

Woloszynska-Read, A., Johnson, C.S., and Trump, D.L. (2011). Vitamin D and Cancer: clinical aspects. *Best Pract. Res. Clin. Endocrinol. Metab.* 25(4), 605-15.

Woods, A., Johnstone, S.R., Dickerson, K., Leiper, F.C., Fryer, L.G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M., and Carling, D. (2003). LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr. Biol.*13, 2004–2008.

Woods, A., Dickerson, K., Heath, R., Hong, S.P., Momcilovic, M., Johnstone, S.R., Carlson, M., and Carling, D. (2005). C(Ca²⁺)/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. *Cell. Metab.* 2, 21–33.

Wullschlegel, S., Loewith, R., and Hall, M.N. (2006). TOR signaling in growth and metabolism. *Cell*. 124, 471–484.

Wu, W., Beilhartz, G., Roy, Y., Richard, C.L., Curtin, M., Brown, L., Cadieux, D., Coppolino, M., Farach-Carson, M.C., Nemere, I., and Meckling, K.A. (2010). Nuclear translocation of the 1,25D3 MARRS (membrane associated rapid response to steroids) receptor protein and NF!B in differentiating NB4 leukemia cells. *Exp. Cell. Res.* 316(7), 1101-1108.

Xiao, B., Sanders, M.J., Underwood, E., Heath, R., Mayer, F.V., Carmena, D., Jing, C., Walker, P.A., Eccleston, J.F., Haire, L.F, Saiu, P., Howell, S.A., Aasland, R., Martin, S.R., Carling, D., and Gamblin, S.J. (2011)Structure of mammalian AMPK and its regulation by ADP. *Nature*. 472(7342), 230–233.

Yang, W.L., Perillo, W., Liou, D., Marambaud, P., and Wang, P. (2012). AMPK inhibitor compound C suppresses cell proliferation by induction of apoptosis and autophagy in human colorectal cancer cells. *J. Surg. Oncol.* 106(6), 680-8.

Yang, Y.F., Wu, C.C., Chen, W.P., Chen, Y.L., Su, M.J. (2011). Prazosin induces p53-mediated autophagic cell death in H9C2 cells. *Naunyn Schmiedeberg's Arch Pharmacol* 384(2), 209-16.

Yang, Z.J., Chee, C.E., Huang, S., and Sinicrope, F. (2011). Autophagy modulation for cancer therapy. *Cancer Biol. Ther.* 11,169–76.

Yikomi, T., Laaksi, I., Lou, Y.R., Martikainen, P., Miettinen, S., Pennanen, P., Purmonen, S., Syvaja, H., Vienonen, A., Tuohimaa, P. (2002). Antiproliferative action of vitamin D. *Vitam Horm.* 64:357-406.

Young, A.R., Narita, M., Ferreira, M., Kirschner, K., Sadaie, M., Darot, J.F., Tavaré, S., Arakawa, S., Shimizu, S., Watt, F.M., and Narita, M. (2009). Autophagy mediates the mitotic senescence transition. *Genes Dev.* 23, 798–803.

Young, A.R., and Narita, M. (2010). Connecting autophagy to senescence in pathophysiology. *Curr. Opin. Cell Biol.* 22,234-240.

Young, M.R., Ihm, J., Lozano, Y., Wright, M.A., and Prechel, M.M. (1995). Treating tumor-bearing mice with vitamin D3 diminishes tumor-induced myelopoiesis and associated immunosuppression, and reduces tumor metastasis and recurrence. *Cancer Immunol. Immunother.* 41(1), 37-45.

Zhang, T.Z., Fan, B., Chen, X., Wang, WJ, Jiao YY, Su GF and Li GY. (2014). *Biochem. Biophysics Res. Commun.* 450(2), 966-72.

Zhang, X.D., Wang, Y., Wu, J.C., Lin, F., Han, R., Han, F., Fukunaga, K., and Qin, Z.H. (2009). Down-regulation of Bcl-2 enhances autophagy activation and cell death induced by mitochondrial dysfunction in rat striatum. *J Neurosci Res.* 87(16), 3600–3610.

Zheng R, Yao Q, Du S, Ren C, Sun Q, Xu Z, Lin X, and Yuan Y. (2014). The status of p53 in cancer cells affects the role of autophagy in tumor radiosensitization. *J. BUON.* 19(2), 336-41.

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M.F., Goodyear, L.J., and Moller, D.E. (2001). Role of AMP-activated protein kinase in mechanism of metformin action.

Zhou, W., Heist, R.S., Liu, G., Park, S., Neuberq, D.S., Wain, J.C., Lynch, T.J., Giovannucci, E., and Christiani, D.C. (2005). Vitamin D is associated with improved survival in early-stage non-small cell lung cancer patients. *Cancer Epidemiol. Biomarkers Prev.* 14(10), 2303–2309.

Zhou, W., Heist, R.S., Liu, G., Asomaning, K., Neuberq, D.S., Hollis, B.W., Wain, J.C., Lynch, T.J., Giovannucci, E., Su, L., and Chrisiani, D.C. (2007). Circulating 25-hydroxyvitamin D levels predict survival in early-stage non-small-cell lung cancer patients. *J. Clin. Oncol.* 25(5), 479–485.

Zhuravel, E., Efanova, O., Shestakova, T., Glushko, N., Mezhev, O., Soldatkina, M., and Pogrebnoy, P. (2010). Administration of vitamin D-3 improves antimetastatic efficacy of cancer vaccine therapy in lewis lung carcinoma. *Exp. Oncol.* 32(1), 33-39,

Zong, W.X., and Moll, U. (2008). p53 in autophagy control. *Cell Cycle.* 2947-2948.

Zeng, X., Yan, T., Schupp, J.E., Seo, Y., and Kinsella, T.J. (2007). DNA mismatch repair initiates 6-thioguanine-induced autophagy through p53 activation in human tumor cells. *Clin. Cancer Res.* 13, 1315–1321.

Vita

Khushboo Sharma was born on February 12, 1986, in Ghaziabad, Uttar Pradesh, and is an Indian citizen. Khushboo graduated from Amity International High School, Noida, India in 2004. She received her Bachelor of Science in Biotechnology from ICFAI-Tech university in Hyderabad, India in May of 2008. She received her Masters of Science in Biomedical Engineering from Virginia Commonwealth University, Virginia in July of 2010. In August of 2010, Khushboo started the PhD program in the department of Pharmacology and Toxicology at Virginia Commonwealth University, Medical College of Virginia campus.

QUALIFICATIONS

- Adept in many science disciplines including biochemistry, molecular biology, biology, carcinogenesis, oncology, toxicology, pharmacology, and radiation oncology
- Outstanding in retrieving interpreting, and analyzing complex data
- Exceptional flexibility and ability to manage simultaneous priorities, changing deadlines, and limited resources
- Sound ability to work independently and take initiative to lead new projects and assignments
- Excellent record of written and oral communication.

EDUCATION

Doctor of Philosophy in Pharmacology and Toxicology

August 2010- Present

Virginia Commonwealth University School of Medicine (In the tradition of the Medical College of Virginia)

Richmond, Virginia

School of Medicine, Department of Pharmacology & Toxicology

Dissertation: A novel cytostatic form of autophagy in sensitization of non-small cell lung cancer cells to radiation by vitamin D and the vitamin D analog, EB 1089.

Masters of Science in Biomedical Engineering

May 2010

Virginia Commonwealth University, Richmond, Virginia

Department of Biomedical Engineering

Thesis: Synthesis and characterization of a novel amphiphilic core-corona hyperbranched polymer composed of EHMO and EHMO-peg, for drug delivery.

WORK EXPERIENCE

Graduate research Associate

2010-2014

Laboratory of Dr. David Gewirtz, PhD Pharmacology and Toxicology department, Virginia Commonwealth University, Massey cancer center.

- Investigating role of vitamin D in the sensitization of NSCLC cells to radiation therapy, specifically evaluating the role of autophagy. Conducted studies to establish role of various chemotherapies (Adriamycin and Camptothecin) in the autophagy-senescence connection in breast tumor cells.
- Apply fundamental pharmacological and molecular concepts, principles and techniques to determine intracellular signaling mechanisms involved in vitamin D mediated radio-sensitization NSCLC.

- Produced written technical material as a primary author on a research manuscript based on my dissertation project and published findings in a peer reviewed research journal, Autophagy. Researched and contributed to other manuscripts those are published on other well-reputed journals.
- Communicated orally to make concise and convincing presentations at numerous seminars in the department. Additionally, I have represented Pharmacology and Toxicology department at national conferences such as AACR (American Association of cancer) annual meetings.
- Provides leadership and guidance to fellow students in the proper use of equipment, conduct of laboratory methods, analysis of data, interpretation of data through journal clubs, and writing of scientific papers.
- Established and maintained effective working relationships with fellow scientists which fostered collaborations in diverse disciplines.

Masters of Science, Research Assistant

2008-2010

Laboratory of Dr. Hu Yang, Ph.D.

Department of Biomedical Engineering

Virginia Commonwealth University, Richmond, Virginia

- Synthesized and characterized a novel amphiphilic core-corona hyperbranched polymer for anti-cancer drug delivery
- Conducted research using various chemical and molecular tools
- Gained hands on experience with techniques like HPLC, DLS (Dynamic Light Scattering), DSC (Differential Scanning Calorimetry), NMR (Nuclear Magnetic Resonance), and SEM (Scanning Electron Microscopy)
- Primary author on a manuscript, submitted to a well-reputed journal. Researched and contributed to other manuscripts as well.

PUBLICATIONS

Sharma K, Goehe RW, Di X, Hicks MA II, Torti SV, Torti FM, Harada H, and Gewirtz DA. A novel cytostatic form of autophagy in sensitization of non-small cell lung cancer cells to radiation by vitamin D and the vitamin D analog, EB 1089. Autophagy. (Accepted, In press).

Sharma K, Le N, Alotaibi M, and Gewirtz DA. Cytotoxic autophagy in cancer therapy. Int J Mol Sci. 2014. 15(6):10034-51.

Sharma K, Goehe RW, Beckta JM, Valerie K, and Gewirtz, DA. Autophagy and radiosensitization in cancer. EXCLI journal. 2014. 13:178-191

Goehe RW, Di X, **Sharma K**, Bristol ML, Henderson SC, Valerie K, Rodier F, Davalos AR, and Gewirtz DA. The autophagy-senescence connection in chemotherapy; Must tumor cells (self) eat before they sleep. J Pharmacol Exp Ther. 2012. 343(3): 763-78.

Sharma K, Zolotarskaya OY, Wynne KJ, and Yang H. Poly (ethyl glycol)-armed hyperbranched polyxetanes for anti-cancer drug delivery. J Bioact Compat Polym. 2012. 27(6):525-539.

MEMBERSHIP-PROFESSIONAL SOCIETIES

American Association for Cancer Research (AACR)	2011- Present
Pharmacology and Toxicology Student Organization Virginia Commonwealth University	2011 – 2014

AWARDS AND HONORS

Thesis/Dissertation Award Virginia Commonwealth University Department of Biomedical Engineering <i>Richmond, VA</i>	January 2010
---	---------------------

CONFERENCES & SYMPOSIUMS

Virginia Academy of Science conference <i>Richmond, VA</i>	May 2014
AACR Conference <i>San Diego, CA</i>	April 2014
Massey Cancer Research Retreat <i>Richmond, VA</i>	June 2013
AACR Conference <i>Washington D.C.</i>	April 2013
2011 Massey Cancer Research Retreat <i>Richmond, VA</i>	November 2011
Cancer Center Pharmacology & Toxicology Retreat <i>Williamsburg, VA</i>	October 2011
Daniel T. Watts Research Poster Symposium. Virginia Commonwealth University <i>Richmond, Virginia</i>	October 2011